



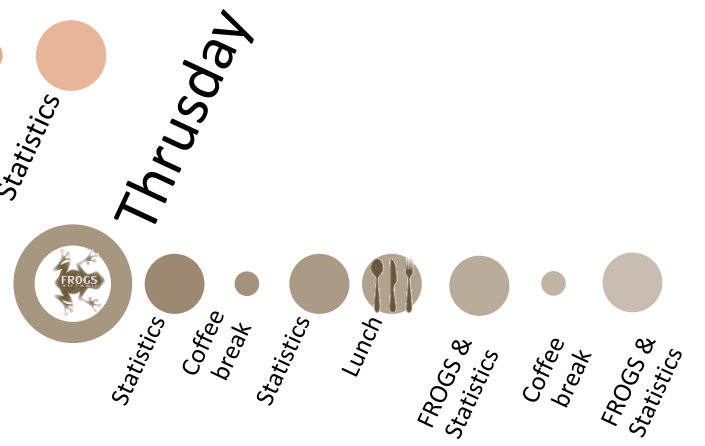
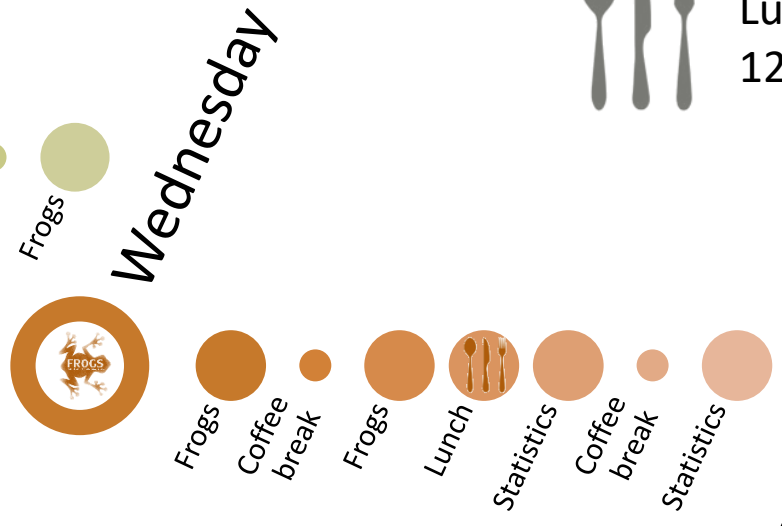
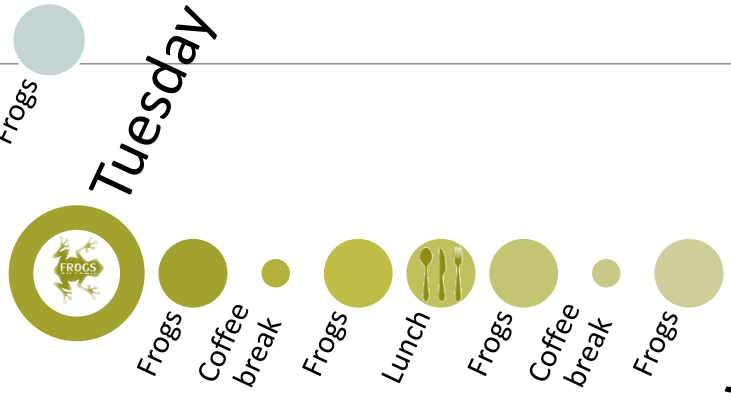
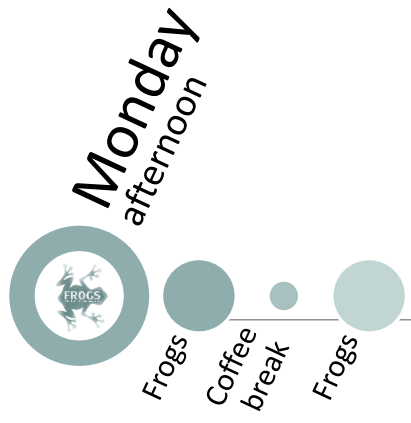
Training on Galaxy: Metagenomics

July 2019

Find, Rapidly, OTUs with Galaxy Solution

FRÉDÉRIC EscUDIÉ* and LUCAS AUER*, MARIA BERNARD, LAURENT CAUQUIL, SARAH MAMAN, MAHENDRA MARIADASSOU, SYLVIE COMBES, GUILLERMINA HERNANDEZ-RAQUET, GÉRALDINE PASCAL

*THESE AUTHORS HAVE CONTRIBUTED EQUALLY TO THE PRESENT WORK.



9 am to 5 pm



2 short coffee breaks
morning and afternoon



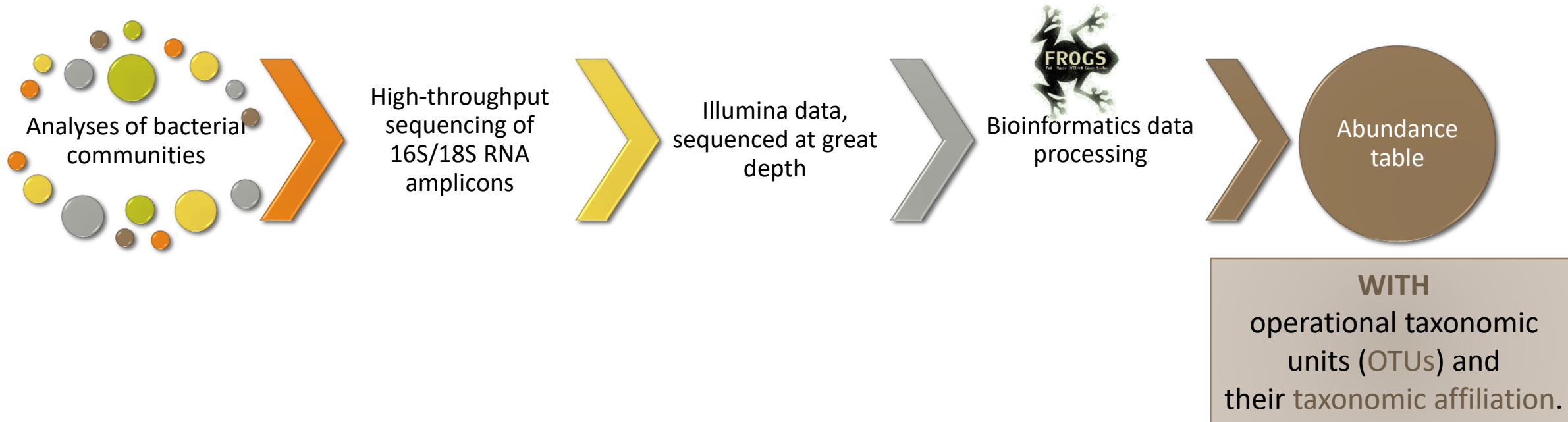
Lunch
12.30 to 2.00 pm

Overview



-
- Objectives
 - Material: data + FROGS
 - Demultiplex tool
 - Preprocessing
 - Clustering + Cluster Statistics
 - Chimera removal
 - Filtering
 - Affiliation + Affiliation Statistics
 - Normalization
 - Tool descriptions
 - Format transformation
 - Export your data
 - Some figures
 - ITS analysis
 - Workflow creation

Objectives



OTUs for ecology

Operational Taxonomy Unit:

a grouping of similar sequences that can be treated as a single « species »

Strengths:

- Conceptually simple
- Mask effect of poor quality data
 - Sequencing error
 - In vitro recombination (chimera)

Weaknesses:

- Limited resolution
- Logically inconsistent definition

Objectives

	Affiliation	Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6
OTU1	Species A	0	100	0	45	75	18645
OTU2	Species B	741	0	456	4421	1255	23
OTU3	Species C	12786	45	3	0	0	0
OTU4	Species D	127	4534	80	456	756	108
OTU5	Species E	8766	7578	56	0	0	200

Why FROGS was developed ?

The **current processing** pipelines **struggle** to run in a reasonable time.

The most effective solutions are often **designed for specialists** making access difficult for the whole community.

In this context we developed the pipeline FROGS: « Find Rapidly OTU with Galaxy Solution ».

Who is in the FROGS group?



Maria BERNARD



Olivier RUÉ



Frédéric ESCUDIÉ



Lucas AUER



**Laurent
CAUQUIL**



**Sylvie
COMBES**



**Guillermina
HERNANDEZ-RAQUET**



Sarah MAMAN

Developers

Biology experts

Galaxy
support



**Mahendra
MARIADASSOU**

Statistical expert



**Géraldine
PASCAL**

Coordinator

Who is in the FROGS group?



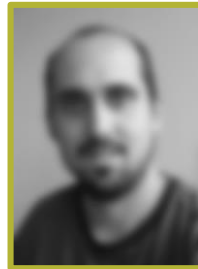
Maria BERNARD



Olivier RUÉ



Frédéric ESCUDIÉ



Lucas AUER



**Laurent
CAUQUIL**



**Sylvie
COMBES**



**Guillermina
HERNANDEZ-RAQUET**



Sarah MAMAN

Developers

Biology experts

Galaxy
support



**Mahendra
MARIADASSOU**

Statistical expert

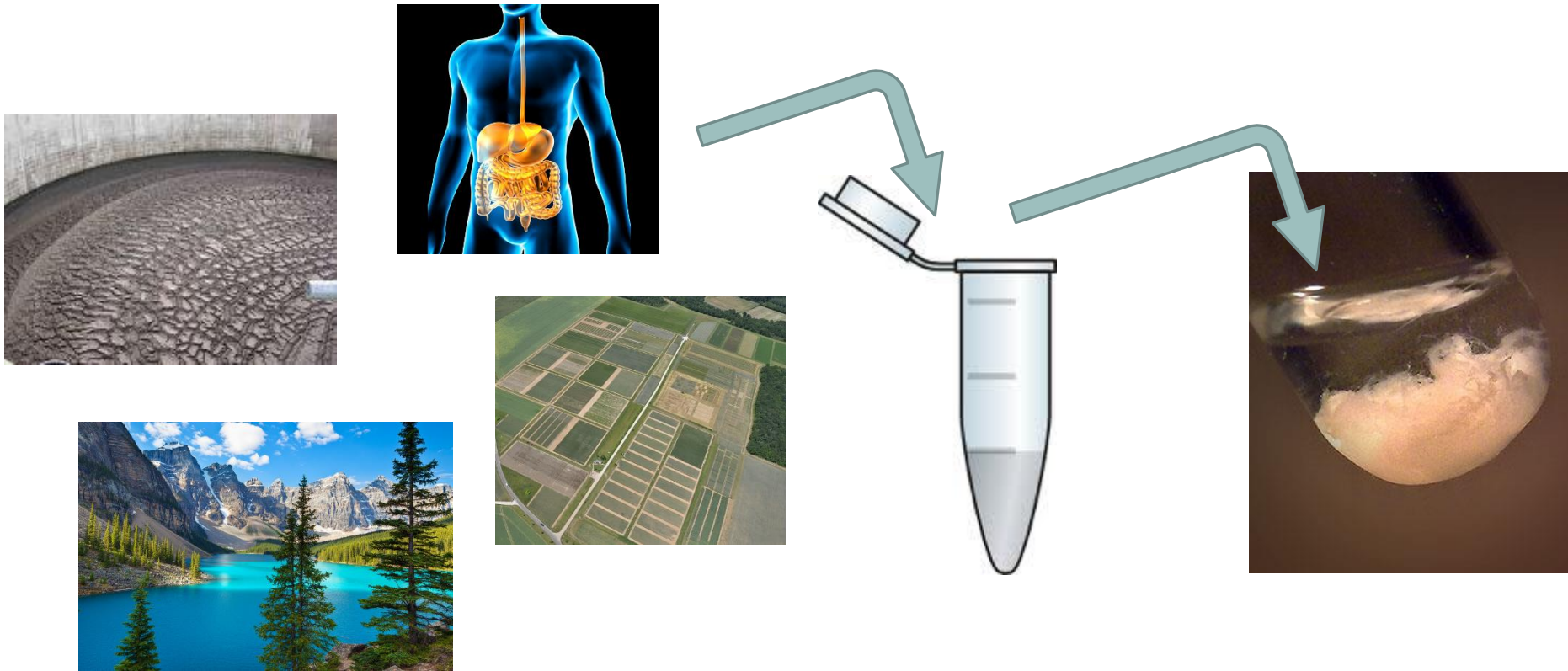


**Géraldine
PASCAL**

Coordinator

Material

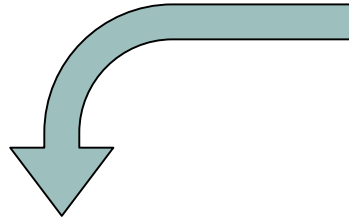
Sample collection and DNA extraction



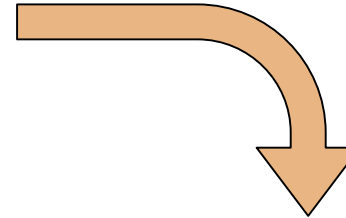
« Meta-omics » using next-generation sequencing (NGS)



DNA



RNA



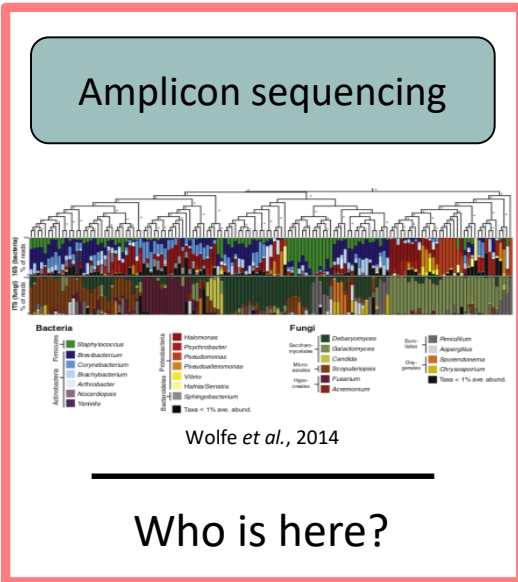
Metagenomics

Metatranscriptomics

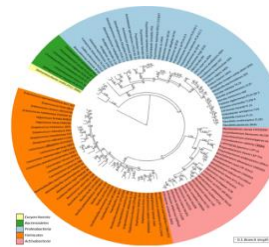
Amplicon sequencing

Shotgun sequencing

RNA sequencing

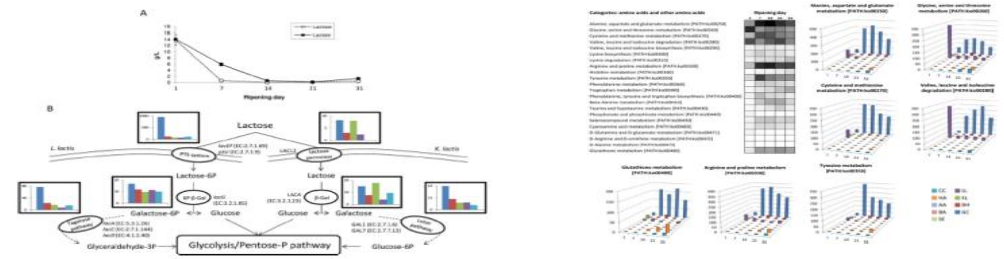


Who is here?



Almeida et al., 2014

What can they do?



Dugat-Bony et al., 2015

What are they doing?

The gene encoding the small subunit of the ribosomal RNA

The most widely used gene in **molecular phylogenetic** studies

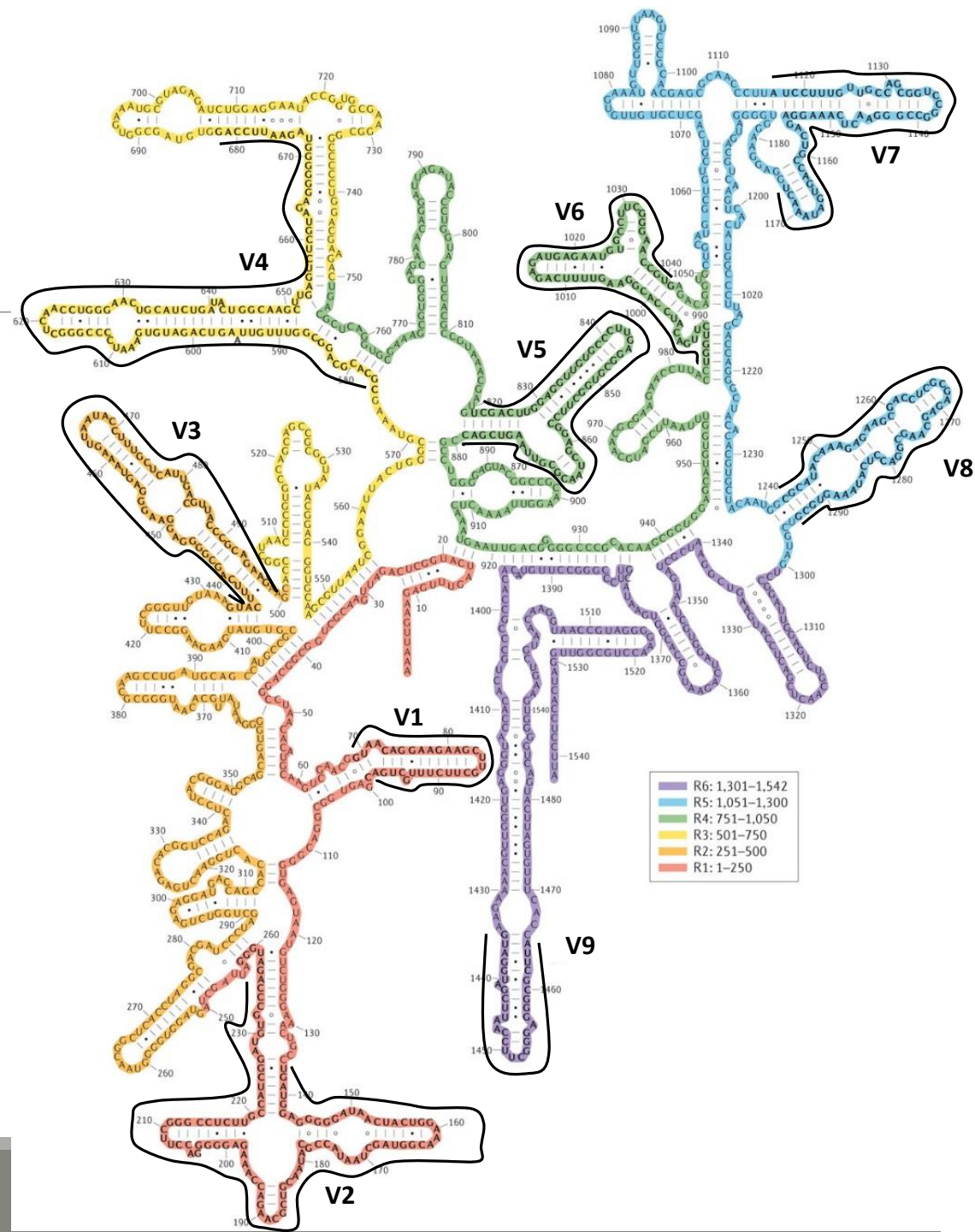
Ubiquist gene : **16S rDNA** in prokaryotes ; **18S rDNA** in eukaryotes

Gene encoding a ribosomal RNA : non-coding RNA (not translated), part of the small subunit of the ribosome which is responsible for the translation of mRNA in proteins

Not submitted to lateral gene transfer

Availability of databases facilitating comparison

(Silva v132 2017: available SSU/LSU sequences to over **6,800,000**)



Secondary structure of the 16S rRNA of *Escherichia coli*

In red, fragment R1 including regions V1 and V2;
 in orange, fragment R2 including region V3;
 in yellow, fragment R3 including region V4;
 in green, fragment R4 including regions V5 and V6;
 in blue, fragment R5 including regions V7 and V8;
 and in purple, fragment R6 including region V9.

Uniting the classification of cultured and uncultured bacteria and archaea using 16S rRNA gene sequences
 Pablo Yarza, et al.
 Nature Reviews Microbiology 12, 635-645
 (2014) doi:10.1038/nrmicro3330

The gene encoding the small subunit of the ribosomal RNA



CONSERVED REGIONS: unspecific applications

VARIABLE REGIONS: group or species-specific applications

Other targets

Bacterial lineages vary in their genomic contents, which suggests that different genes might be needed to resolve the diversity within certain taxonomic groups.

The genes that have been proposed for this task include those encoding :

- 23S rRNA,
- DNA gyrase subunit B (*gyrB*),
- RNA polymerase subunit B (*rpoB*),
- TU elongation factor (*tuf*),
- DNA recombinase protein (*recA*),
- protein synthesis elongation factor-G (*fusA*),
- dinitrogenase protein subunit D (*nifD*),
- Internal Transcribed Spacer (ITS) for Fungi.

Other targets

- *gyrB* has a higher rate of base substitution than 16S rDNA does, and shows promise for community-profiling applications.
- This gene is essential and ubiquitous in bacteria and
- is sufficiently large in size for use in analysis of microbial communities.
- It is a single-copy housekeeping gene that encodes the subunit B of DNA gyrase, a type II DNA topoisomerase, and therefore plays an essential role in DNA replication.
- Furthermore, the *gyrB* gene is also present in Eukarya and sometimes in Archaea but it shows enough sequence dissimilarity between the three domains of life to be used selectively for Bacteria.

Other target

See for *gyrB* :

Article of Stéphane Chaillou

RESEARCH ARTICLE

Deciphering intra-species bacterial diversity of meat and seafood spoilage microbiota using *gyrB* amplicon sequencing: A comparative analysis with 16S rDNA V3-V4 amplicon sequencing

Simon Poirier¹, Olivier Rué², Raphaëlle Peguignan¹, Gwendoline Coeuret¹, Monique Zagorec³, Marie-Christine Champomier-Vergès¹, Valentin Loux², Stéphane Chaillou^{1*}

1 MICALIS, INRA, AgroParisTech, Université Paris-Saclay, Jouy-en-Josas, France, **2** MaIAGE, INRA, Université Paris-Saclay, Jouy-en-Josas, France, **3** Secalim, INRA, Oniris, Nantes, France

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OPEN ACCESS

Citation: Poirier S, Rué O, Peguignan R, Coeuret G, Zagorec M, Champomier-Vergès M-C, et al. (2018) Deciphering intra-species bacterial diversity of meat and seafood spoilage microbiota using *gyrB* amplicon sequencing: A comparative analysis with 16S rDNA V3-V4 amplicon sequencing. PLoS ONE 13(9): e0204629. <https://doi.org/10.1371/journal.pone.0204629>

Editor: George-John Nychas, Agricultural University of Athens, GREECE

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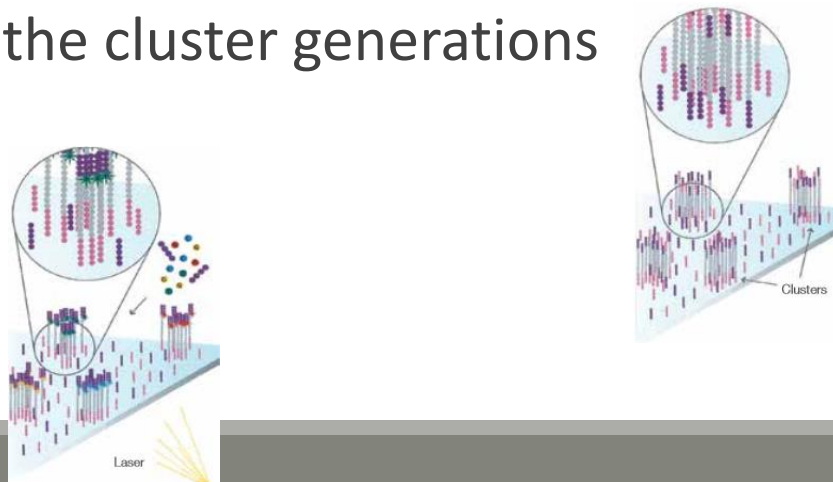
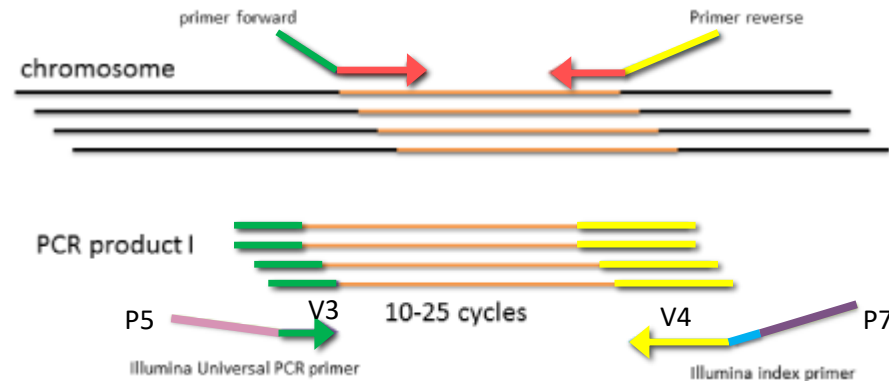
Data Availability Statement: Raw read sequences were deposited at the Sequence Read Archive under the accession numbers SAMN09070427 to SAMN09070506. The whole dataset has been uploaded to figshare and is accessible using the following DOI: [10.6084/m9.figshare.7083209](https://doi.org/10.6084/m9.figshare.7083209). The R script (`redlosses_phyloseq_custom.R`), which includes all commands performed to create our figures, is available for download at DOI: [10.6084/m9.figshare.7083254](https://doi.org/10.6084/m9.figshare.7083254).

Abstract

Meat and seafood spoilage ecosystems harbor extensive bacterial genomic diversity that is mainly found within a small number of species but within a large number of strains with different spoilage metabolic potential. To decipher the intraspecies diversity of such microbiota, traditional metagenetic analysis using the 16S rRNA gene is inadequate. We therefore assessed the potential benefit of an alternative genetic marker, *gyrB*, which encodes the subunit B of DNA gyrase, a type II DNA topoisomerase. A comparison between 16S rDNA-based (V3-V4) amplicon sequencing and *gyrB*-based amplicon sequencing was carried out in five types of meat and seafood products, with five mock communities serving as quality controls. Our results revealed that bacterial richness in these mock communities and food samples was estimated with higher accuracy using *gyrB* than using 16S rDNA. However, for *Firmicutes* species, 35% of putative *gyrB* reads were actually identified as sequences of a *gyrB* paralog, *parE*, which encodes subunit B of topoisomerase IV; we therefore constructed a reference database of published sequences of both *gyrB* and *parE* for use in all subsequent analyses. Despite this co-amplification, the deviation between relative sequencing quantification and absolute qPCR quantification was comparable to that observed for 16S rDNA for all the tested species. This confirms that *gyrB* can be used successfully alongside 16S rDNA to determine the species composition (richness and evenness) of food microbiota. The major benefit of *gyrB* sequencing is its potential for improving taxonomic assignment and for further investigating OTU richness at the subspecies level, thus allowing more accurate discrimination of samples. Indeed, 80% of the reads of the 16S rDNA dataset were represented by thirteen 16S rDNA-based OTUs that could not be assigned at the species-level. Instead, these same clades corresponded to 44 *gyrB*-based OTUs, which differentiated various lineages down to the subspecies level. The increased ability of *gyrB*-based analyses to track and trace phylogenetically different groups of strains

Steps for Illumina sequencing

- 1st step : one PCR
- 2nd step: one PCR
- 3rd step: on flow cell, the cluster generations
- 4th step: sequencing



Amplification and sequencing

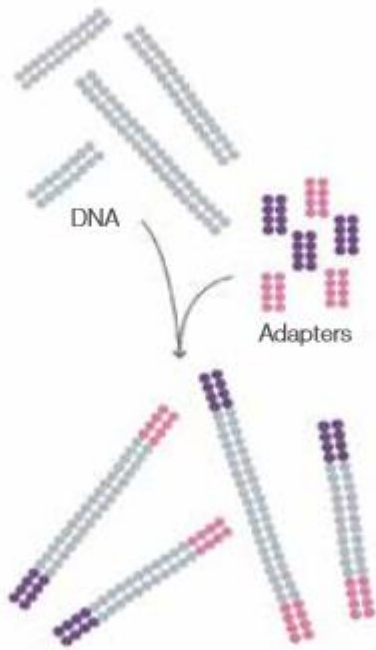
« **Universal** » primer sets are used for **PCR amplification** of the phylogenetic biomarker

The primers contain **adapters** used for the sequencing step and **barcodes** (= tags = MIDs) to distinguish the samples (multiplexing = sequencing several samples on the same run)



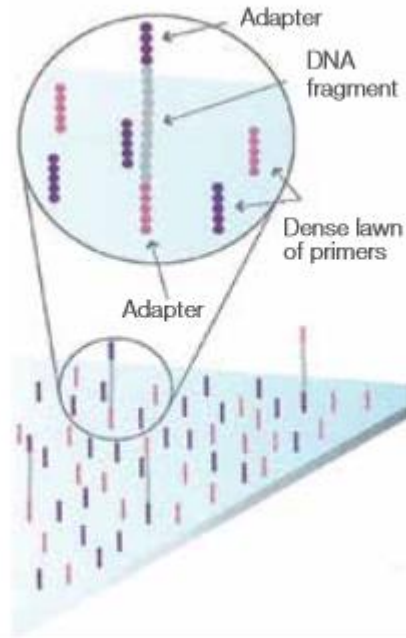
Cluster generation

Prepare Genomic DNA Sample



Randomly fragment genomic DNA and ligate adapters to both ends of the fragments.

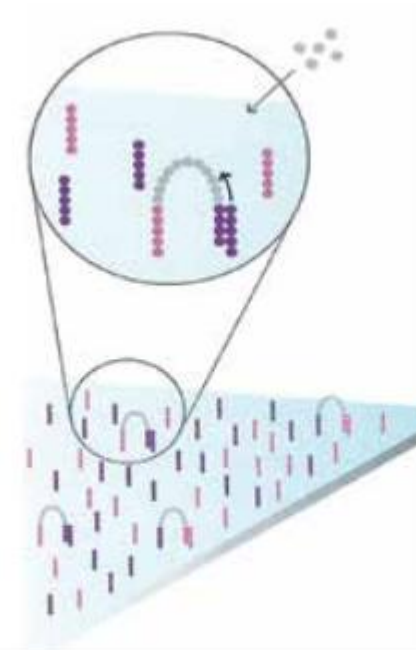
Attach DNA to Surface



Bind single-stranded fragments randomly to the inside surface of the flow cell channels.

Attach DNA to surface

Bridge Amplification

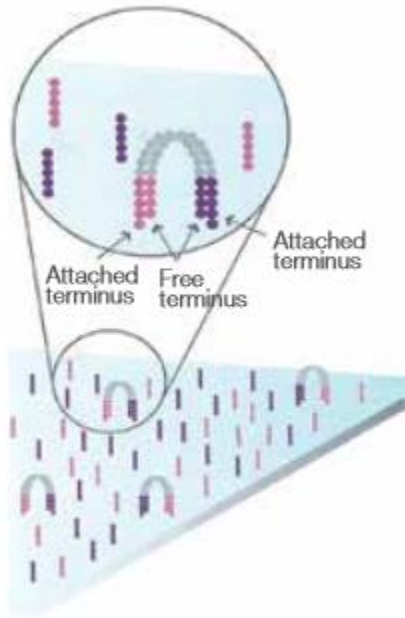


Add unlabeled nucleotides and enzyme to initiate solid-phase bridge amplification.

Bridge amplification

Cluster generation

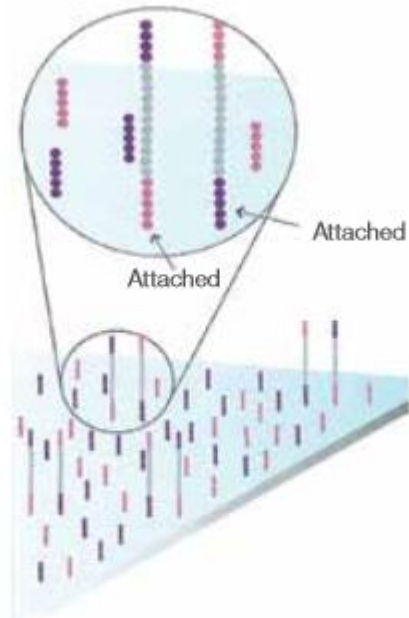
Fragments Become Double Stranded



The enzyme incorporates nucleotides to build double-stranded bridges on the solid-phase substrate.

Fragments become double stranded

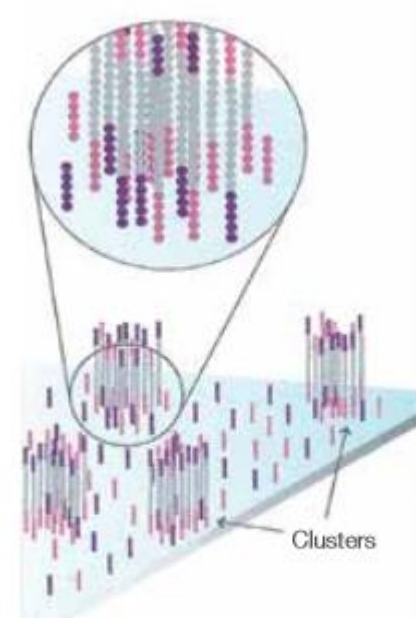
Denature the Double-Stranded Molecules



Denaturation leaves single-stranded templates anchored to the substrate.

Denature the double-stranded molecule

Complete Amplification

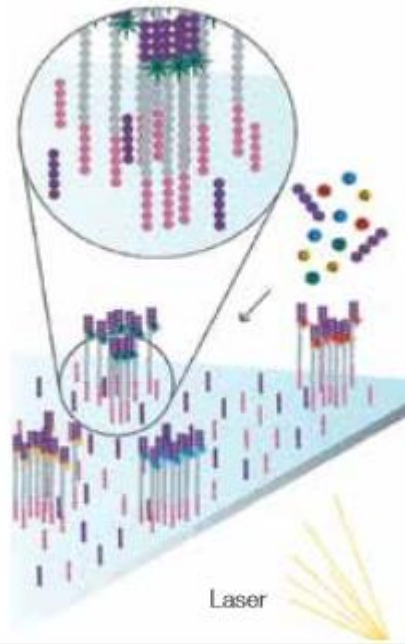


Several million dense clusters of double-stranded DNA are generated in each channel of the flow cell.

Cycle of new strand synthesis and denaturation to make multiple copies of the same sequence (amplification)
Reverse strands are washed

Sequencing by synthesis

Determine First Base



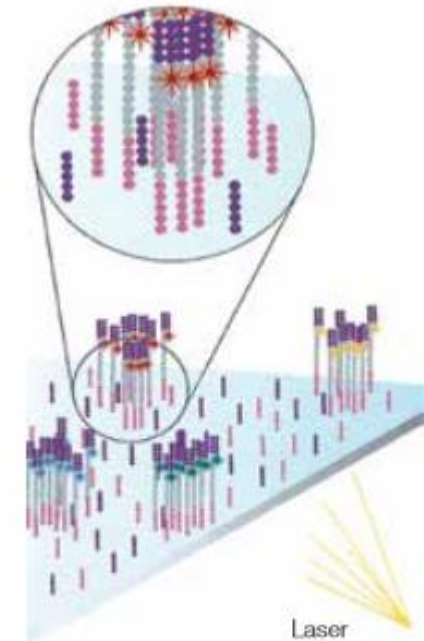
The first sequencing cycle begins by adding four labeled reversible terminators, primers, and DNA polymerase.
Light signal is more strong in cluster

Image First Base



After laser excitation, the emitted fluorescence from each cluster is captured and the first base is identified.

Determine Second Base



The next cycle repeats the incorporation of four labeled reversible terminators, primers, and DNA polymerase.

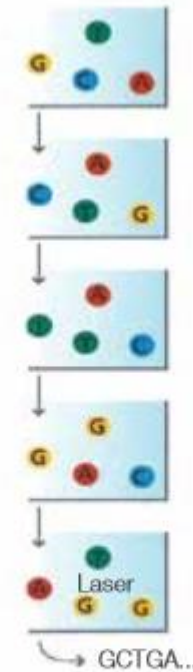
Sequencing by synthesis

Image Second Chemistry Cycle



After laser excitation, the image is captured as before, and the identity of the second base is recorded.

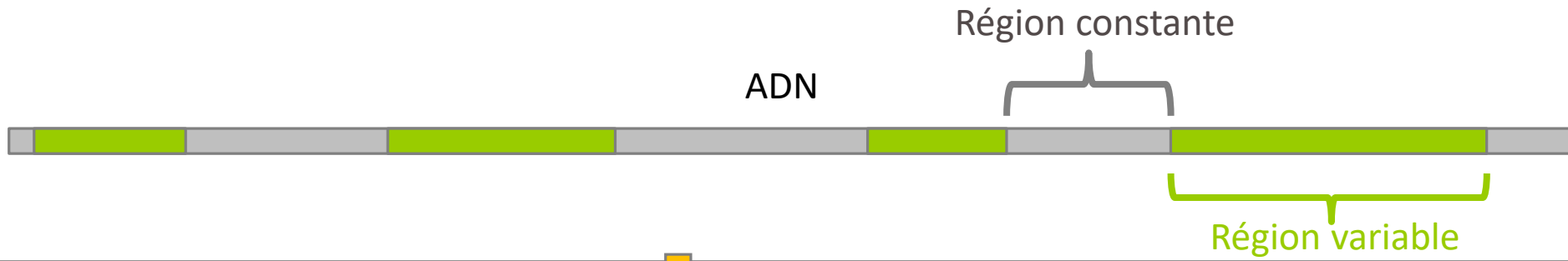
Sequencing Over Multiple Chemistry Cycles



The sequencing cycles are repeated to determine the sequence of bases in a fragment, one base at a time.

Barcode is read, so cluster is identified.

After first sequencing (250 or 300 nt of Reverse strand), fragment form bridges again and Forward strand can be sequenced also.



↓ PCRs

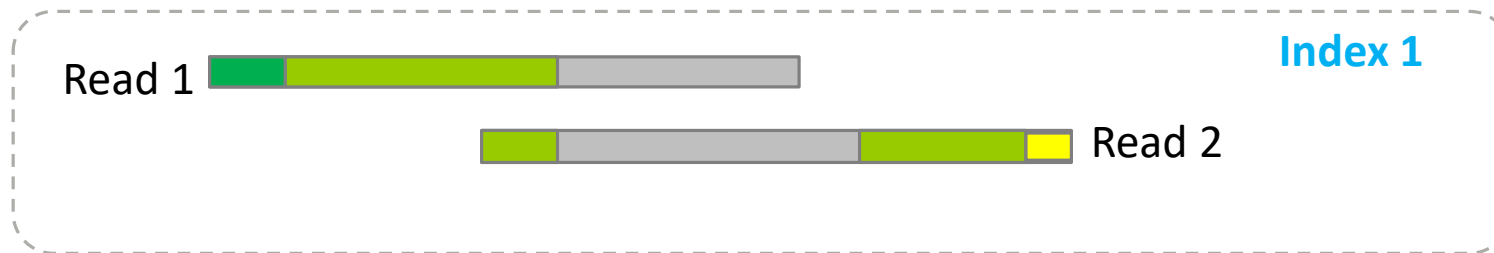
Index Illumina



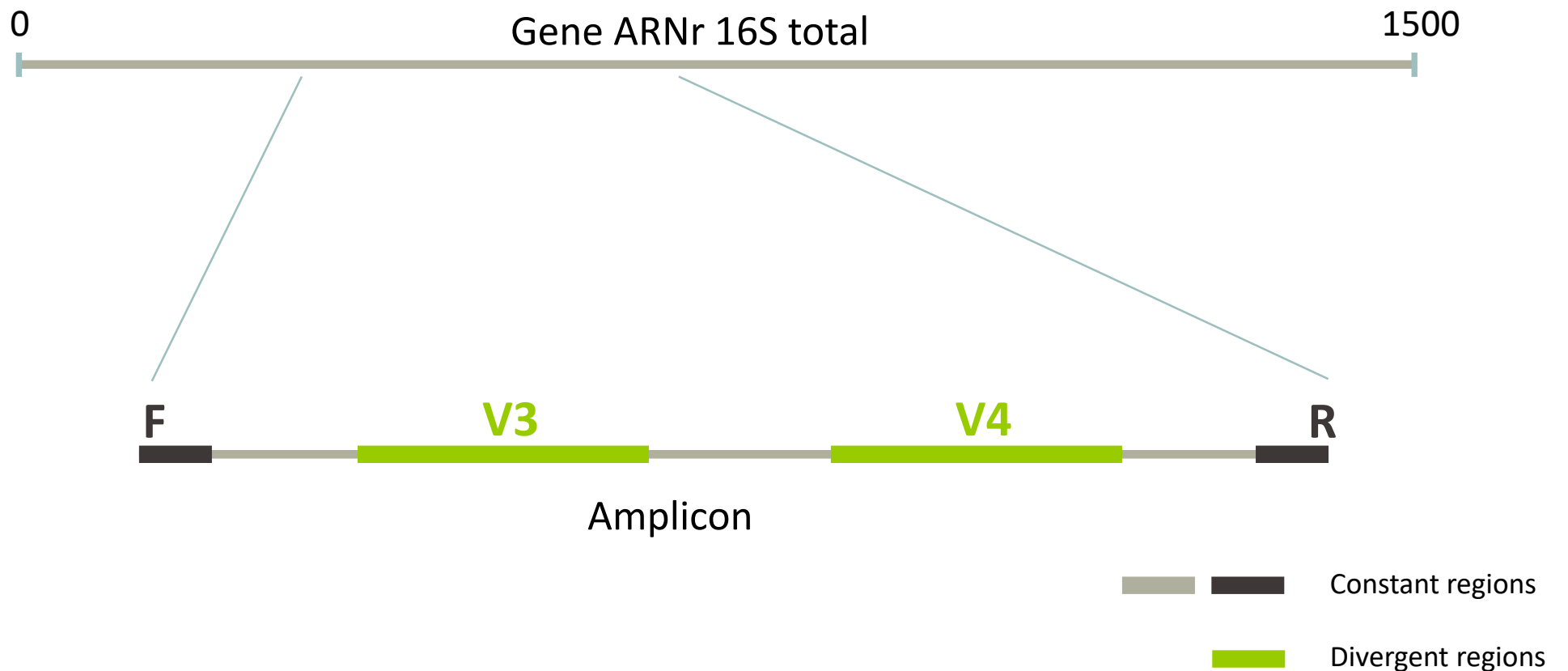
Adaptateur Illumina

Adaptateur Illumina

↓ Séquençage



Identification of bacterial populations may be not discriminating



Amplification and sequencing

Sequencing is generally performed on **Roche-454** (obsolete now) or **Illumina MiSeq** platforms.

Roche-454 generally produce ~ 10 000 reads per sample

MiSeq ~ 30 000 reads per sample

Sequence length is **>650 bp** for pyrosequencing technology (Roche-454) and **2 x 250 bp or 2 x 300 bp** for the MiSeq technology in paired-end mode.

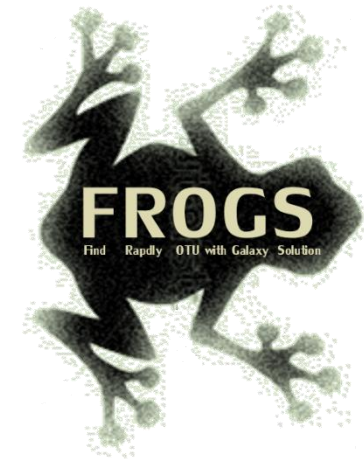


Methods



Which bioinformatics solutions ?

	Disadvantages
QIIME	Installation problem Command lines
UPARSE	Global clustering command lines
MOTHUR	Not MiSeq data without normalization Global hierarchical clustering Command lines
MG-RAST	No modularity No transparency



QIIME allows analysis of high-throughput community sequencing data

J Gregory Caporaso et al, *Nature Methods*, 2010; doi:10.1038/nmeth.f.303

Introducing mothur: Open-source, platform-independent, community-supported software for describing and comparing microbial communities.

Schloss, P.D., et al., *Appl Environ Microbiol*, 2009, doi: 10.1128/AEM.01541-09

UPARSE: Highly accurate OTU sequences from microbial amplicon reads

Edgar, R.C. et al, *Nature Methods*, 2013, dx.doi.org/10.1038/nmeth.2604

The metagenomics RAST server – a public resource for the automatic phylogenetic and functional analysis of metagenomes

F Meyer et al, *BMC Bioinformatics*, 2008, doi:10.1186/1471-2105-9-386

FROGS ?

Use platform **Galaxy**

Set of **modules** = Tools to analyze your “big” data

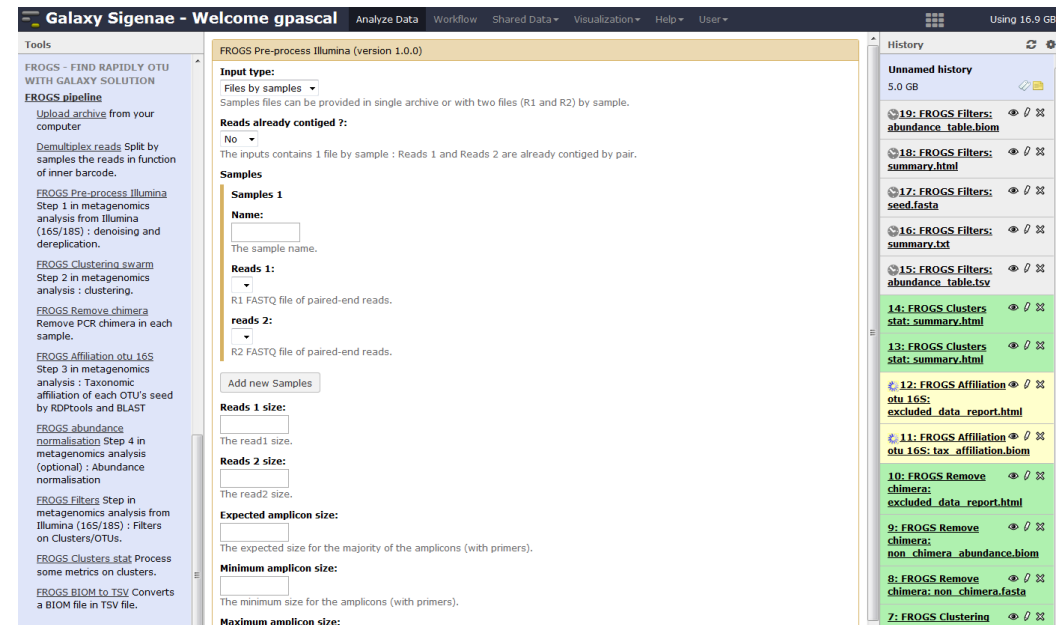
Independent modules

Run on Illumina/454 data **16S, 18S, and 23S, ITS and others**

Innovative clustering method

Many **graphics** for interpretation

User friendly, hiding bioinformatics infrastructure/complexity

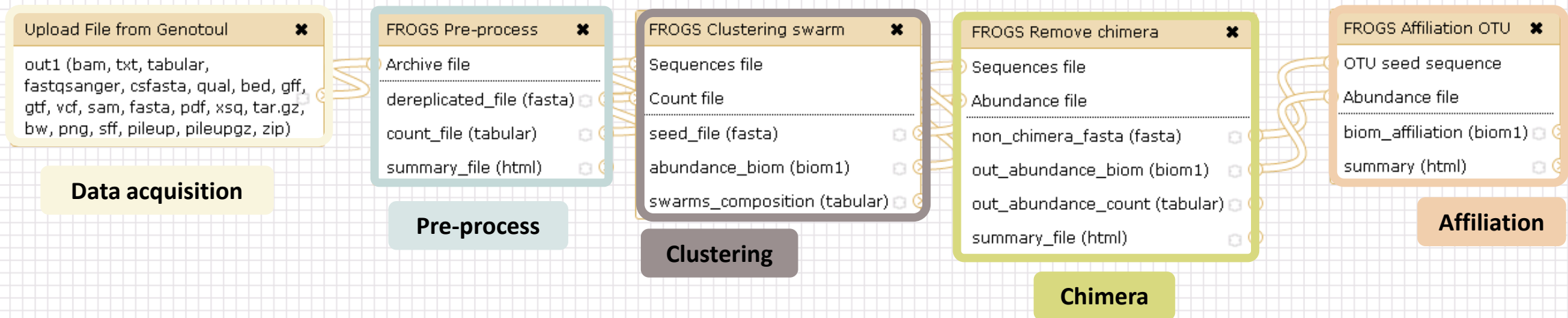


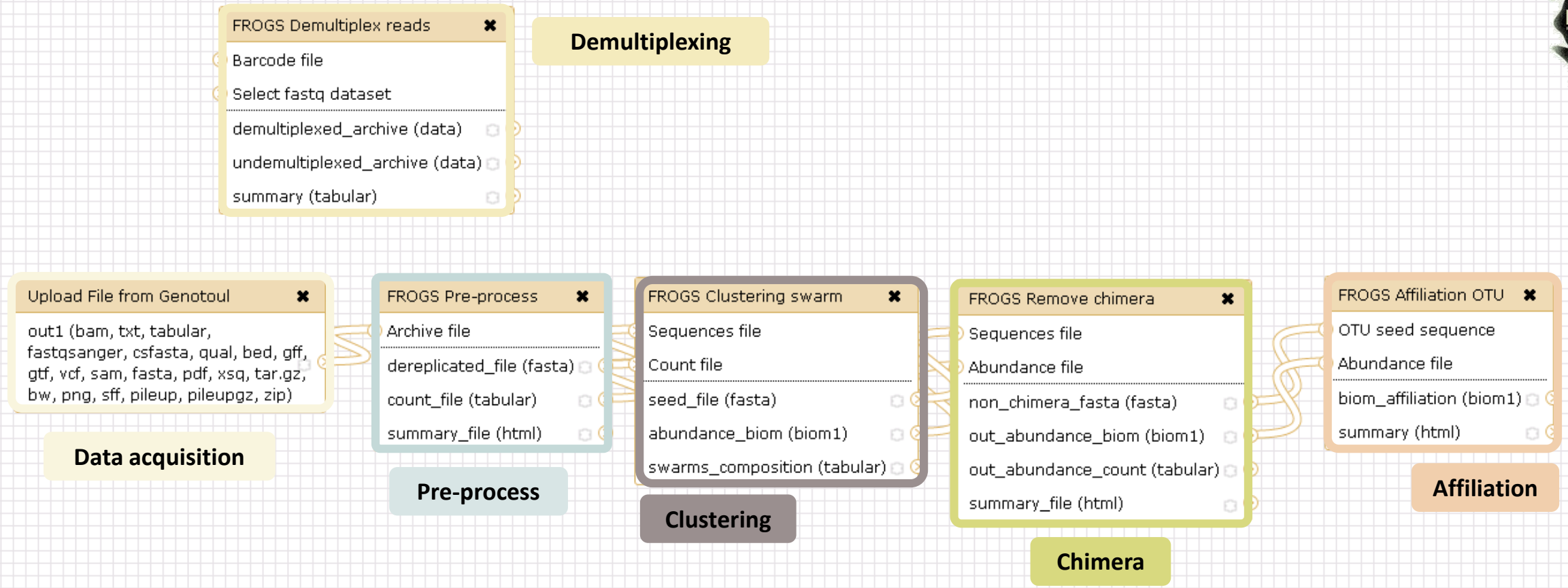
The screenshot displays the Galaxy Sigenae web interface for the 'FROGS Pre-process Illumina (version 1.0.0)' tool. The interface is organized into several panels:

- Tools Panel (Left):** Lists various FROGS modules including 'FROGS - FIND RAPIDLY OTU WITH GALAXY SOLUTION', 'FROGS pipeline', 'Demultiplex reads', 'FROGS Pre-process Illumina', 'FROGS Clustering swarm', 'FROGS Remove chimera', 'FROGS Affiliation otu 16S', 'FROGS abundance normalisation', and 'FROGS Filters'.
- Main Configuration Panel (Center):**
 - Input type:** Set to 'Files by samples'.
 - Reads already contiged?:** Set to 'No'.
 - Samples:** A section for 'Samples 1' with a 'Name' field and a description: 'The sample name.'
 - Reads 1:** A dropdown menu set to 'R1 FASTQ file of paired-end reads.'
 - Reads 2:** A dropdown menu set to 'R2 FASTQ file of paired-end reads.'
 - Buttons:** 'Add new Samples'.
 - Reads 1 size:** A text input field for 'The read1 size.'
 - Reads 2 size:** A text input field for 'The read2 size.'
 - Expected amplicon size:** A text input field for 'The expected size for the majority of the amplicons (with primers).'
 - Minimum amplicon size:** A text input field for 'The minimum size for the amplicons (with primers).'
 - Maximum amplicon size:** A text input field.
- History Panel (Right):** Shows a list of previous jobs, including 'FROGS Filters: abundance_table.biom', 'FROGS Filters: summary.html', 'FROGS Filters: seed.fasta', 'FROGS Filters: summary.txt', 'FROGS Filters: abundance_table.tsv', 'FROGS Clusters stat: summary.html', 'FROGS Clusters stat: summary.html', 'FROGS Affiliation otu 16S: excluded_data_report.html', 'FROGS Affiliation otu 16S: tax_affiliation.biom', 'FROGS Remove chimera: excluded_data_report.html', 'FROGS Remove chimera: non_chimera_abundance.biom', 'FROGS Remove chimera: non_chimera.fasta', and 'FROGS Clustering'.



FROGS Pipeline







FROGS Abundance normalisation ✕

- Sequences file
- Abundance file

output_fasta (fasta)

output_biom (biom1)

summary_file (html)

Normalization

Upload File from Genotoul ✕

out1 (bam, txt, tabular, fastqsanger, csfasta, qual, bed, gff, gtf, vcf, sam, fasta, pdf, xsq, tar.gz, bw, png, sff, pileup, pileupgz, zip)

Data acquisition

FROGS Pre-process ✕

- Archive file
- dereplicated_file (fasta)
- count_file (tabular)
- summary_file (html)

Pre-process

FROGS Clustering swarm ✕

- Sequences file
- Count file
- seed_file (fasta)
- abundance_biom (biom1)
- swarms_composition (tabular)

Clustering

FROGS Remove chimera ✕

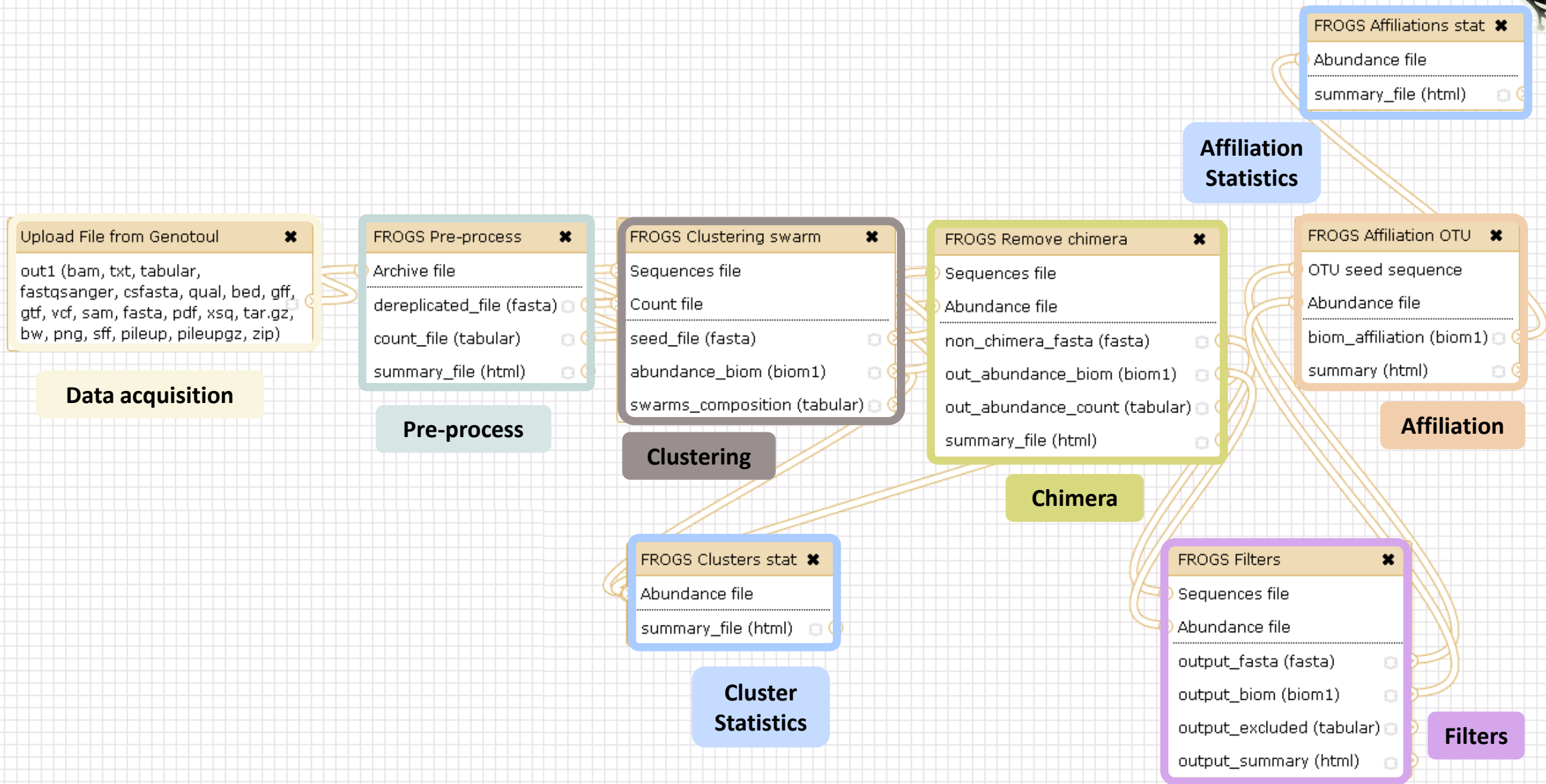
- Sequences file
- Abundance file
- non_chimera_fasta (fasta)
- out_abundance_biom (biom1)
- out_abundance_count (tabular)
- summary_file (html)

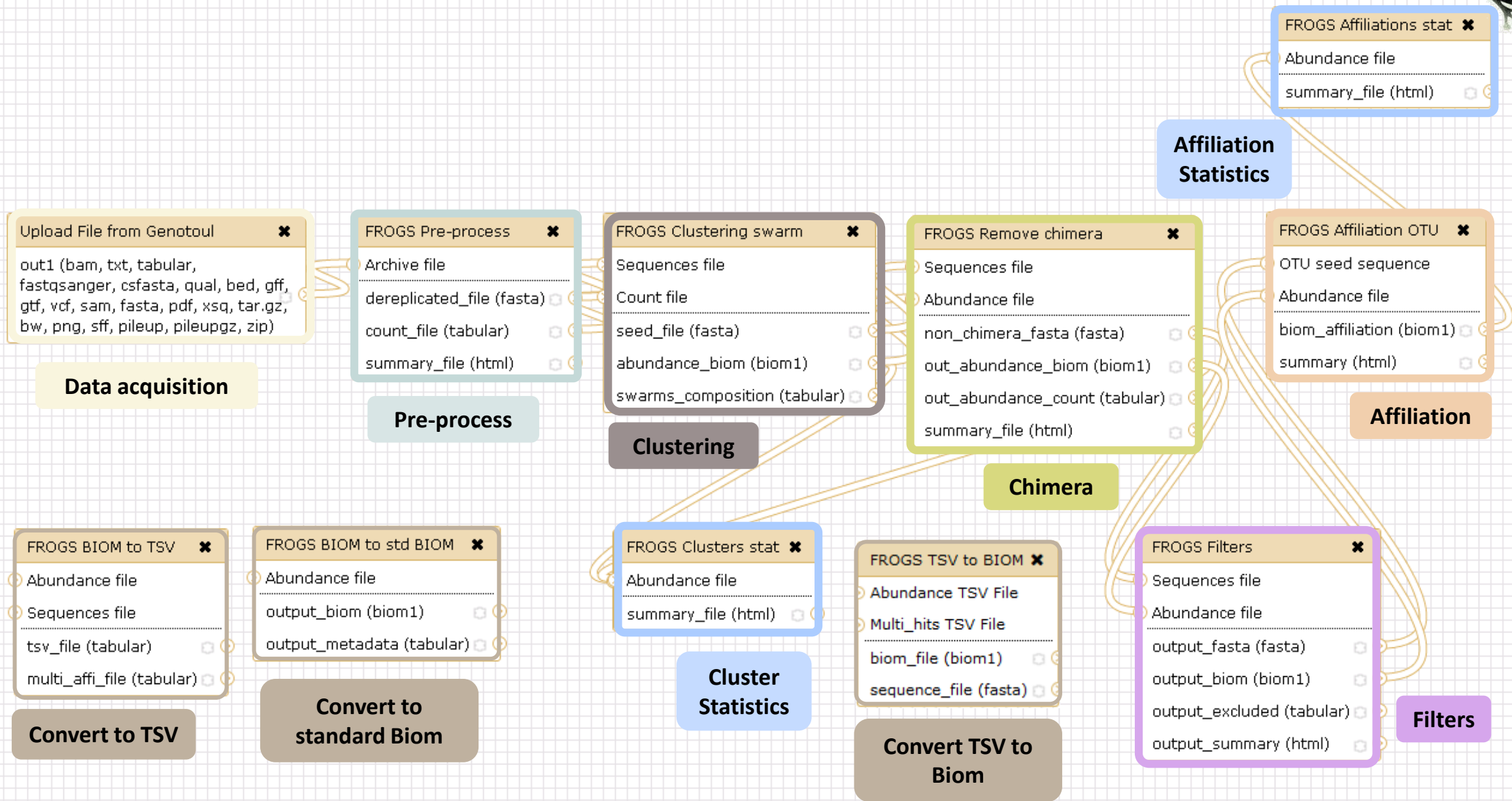
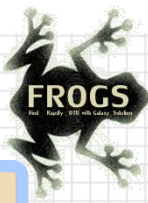
Chimera

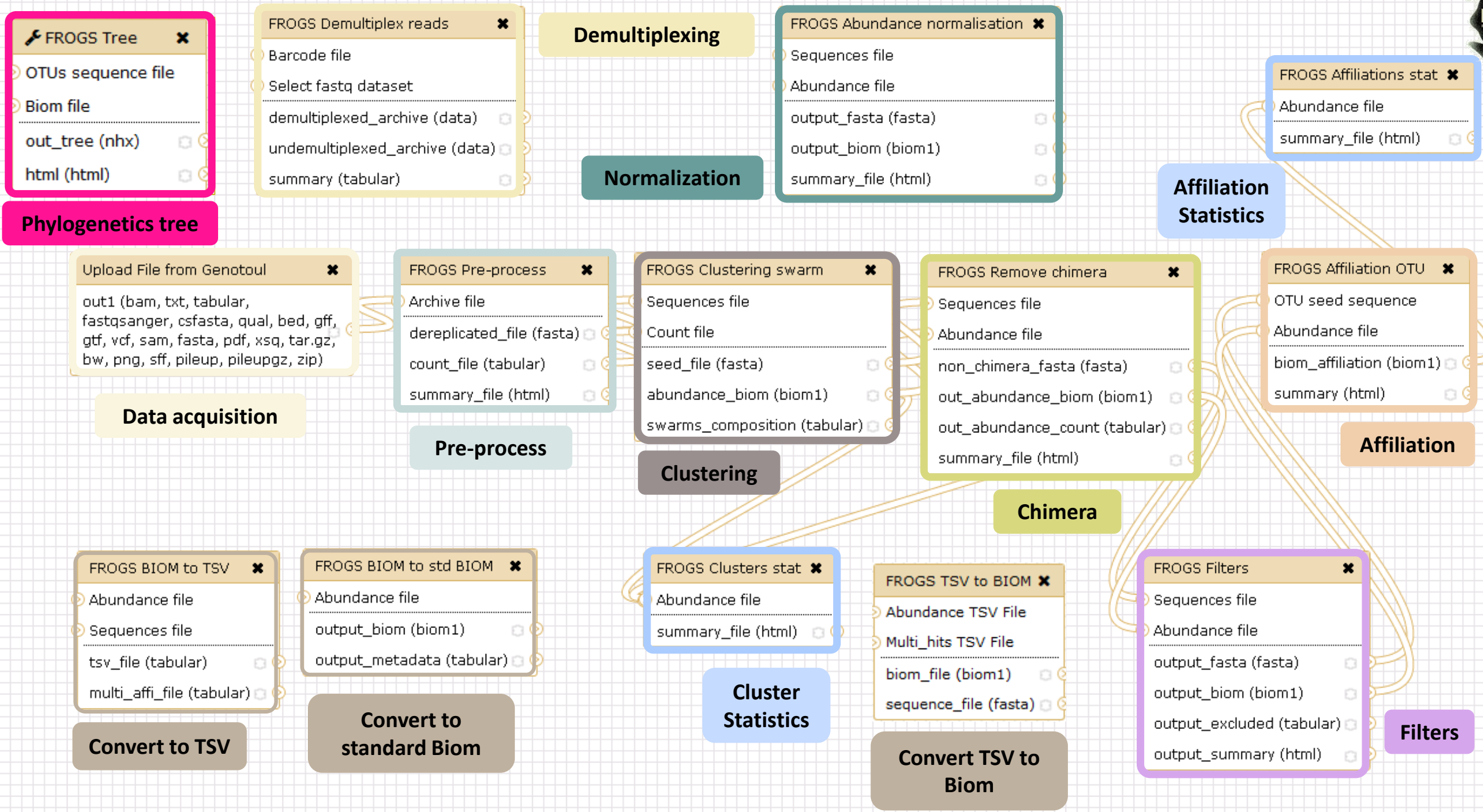
FROGS Affiliation OTU ✕

- OTU seed sequence
- Abundance file
- biom_affiliation (biom1)
- summary (html)

Affiliation



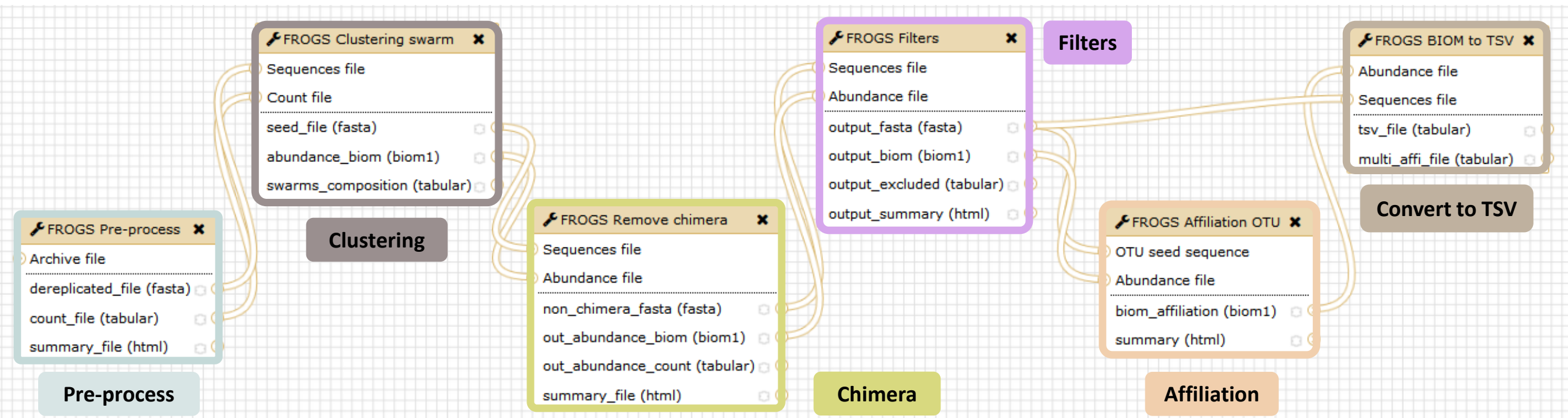


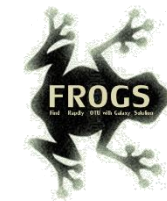




FROGS Pipeline

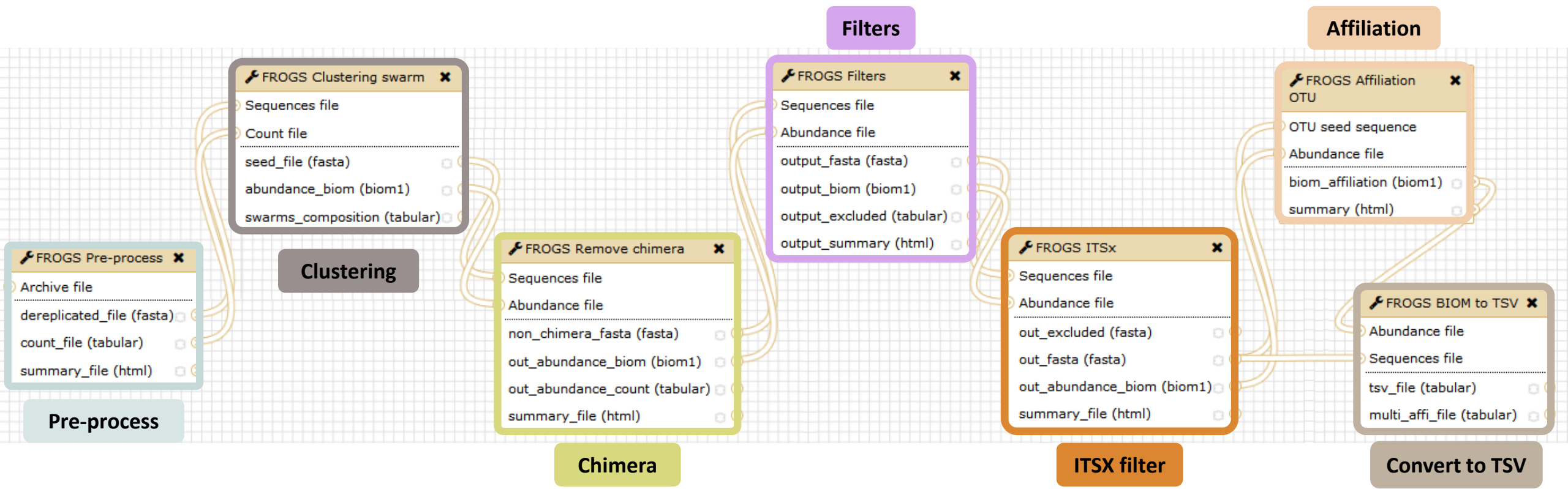
Minimal pipeline for bacterial amplicon analyses





FROGS Pipeline

Minimal pipeline for ITS amplicon analyses



FROGS Tools for Bioinformatics analyses

The screenshot displays the Galaxy web interface. The main panel shows the configuration for the 'FROGS Pre-process Step 1 in metagenomics analysis: denoising and dereplication. (Galaxy Version 1.5.0)'. The configuration includes:

- Sequencer:** Illumina
- Input type:** Files by samples
- Reads already contiged ?** No
- Samples:** 1: Samples (Name field is empty)
- Reads 1:** No fastq dataset available.
- reads 2:** No fastq dataset available.
- Reads 1 size:** (Empty field)
- Reads 2 size:** (Empty field)
- Expected amplicon size:** (Empty field)

The right-hand History panel shows a list of analysis jobs:

- FROGS analysis (444.7 MB)
- 25: FROGS (Affiliations stat: summary.html)
- 24: FROGS BIOM to std BIOM: blast metadata.tsv
- 23: FROGS BIOM to std BIOM: abundance.biom
- 22: FROGS BIOM to TSV: multi_hits.tsv
- 21: FROGS BIOM to TSV: abundance.tsv
- 20: FROGS (Affiliations stat: summary.html)
- 19: FROGS Clusters stat: summary.html
- 18: FROGS Affiliation OTU: report.html
- 17: FROGS Affiliation OTU: affiliation.biom
- 16: FROGS Clusters stat: summary.html
- 15: FROGS Filters: report.html
- 14: FROGS Filters: excluded.tsv
- 13: FROGS Filters: abundance.biom
- 12: FROGS Filters: sequences.fasta

Demultiplexing

Pre-process

Clustering

Chimera

Filters

ITSX

Affiliation

Cluster Stat

Affiliation Stat

Affiliation postprocess

Biom to std Biom

Biom to TSV

TSV to Biom

Normalization

Phylogenetics Tree

Waiting to run

Currently running

Result files

FROGS Tools for Statistic analyses

The screenshot displays the Galaxy web interface. The main panel shows the configuration for the 'FROGS Pre-process' tool. The configuration includes:

- Sequencer:** Illumina
- Input type:** Files by samples
- Reads already contiged ?**: No
- Samples:** 1: Samples
- Reads 1:** No fastq dataset available.
- reads 2:** No fastq dataset available.
- Reads 1 size:** (empty field)
- Reads 2 size:** (empty field)
- Expected amplicon size:** (empty field)

The right-hand panel shows the 'History' section with a list of analysis steps:

- 25: FROGS Affiliations stat: summary.html
- 24: FROGS BIOM to std BIOM: blast_metadata.tsv
- 23: FROGS BIOM to std BIOM: abundance.biom
- 22: FROGS BIOM to TSV: multi_hits.tsv
- 21: FROGS BIOM to TSV: abundance.tsv
- 20: FROGS Affiliations stat: summary.html
- 19: FROGS Clusters stat: summary.html
- 18: FROGS Affiliation OTU: report.html
- 17: FROGS Affiliation OTU: affiliation.biom
- 16: FROGS Clusters stat: summary.html
- 15: FROGS Filters: report.html
- 14: FROGS Filters: excluded.tsv
- 13: FROGS Filters: abundance.biom
- 12: FROGS Filters: sequences.fasta

Import data

Composition visualisation

Alpha diversity

Beta diversity

Structure visualisation

Sample clustering

Multivariate analysis of variance

Waiting to run

Currently running

Result files

What kind of data ?

4 Upload → 4 Histories

Multiplexed data

Pathobiomes
rodents and ticks

`multiplex.fastq`

`barcode_forward.ta
bular`

ITS data

METABARFOOD
project

`ITS.tar.gz`

OU

454 data

Freshwater sediment
metagenome

`454.fastq.gz`

SRA number:
SRR443364

MiSeq

R1 fastq + R2 fastq

Farm animal feces
metagenome

`sampleA_R1.fastq`

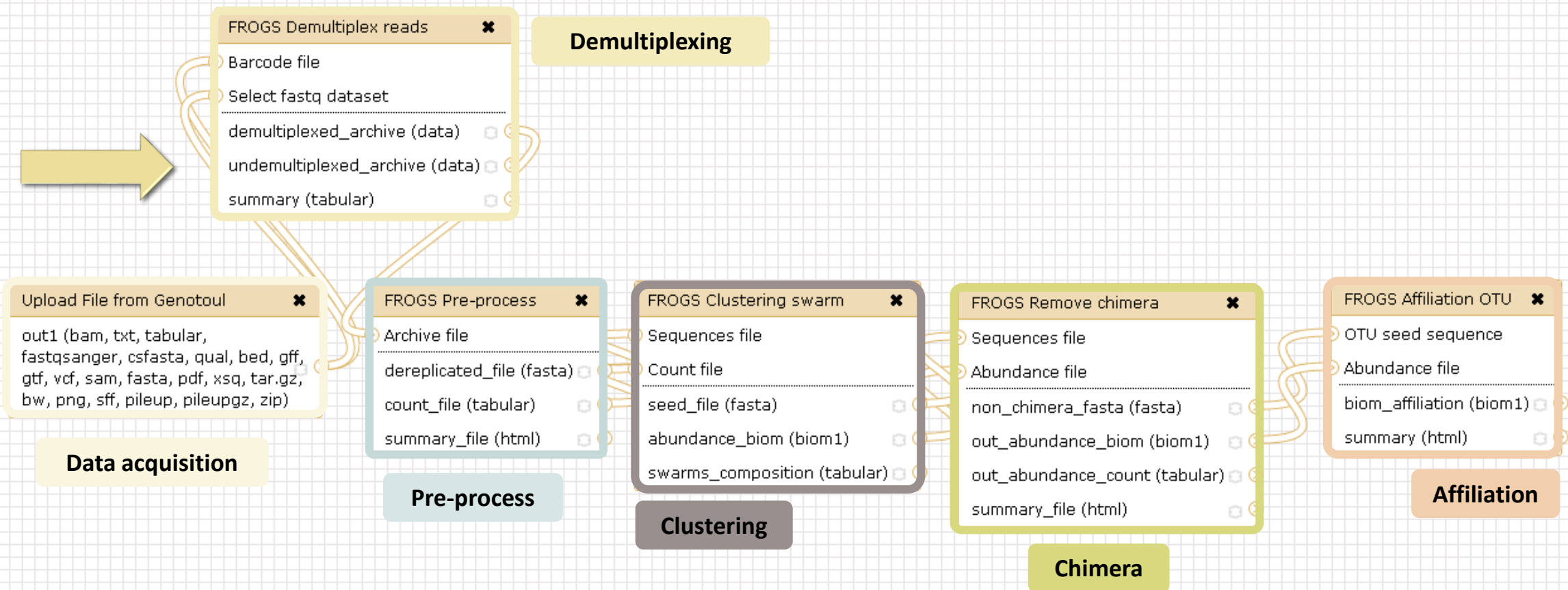
`sampleA_R2.fastq`

MiSeq merged fastq in
archive tar.gz

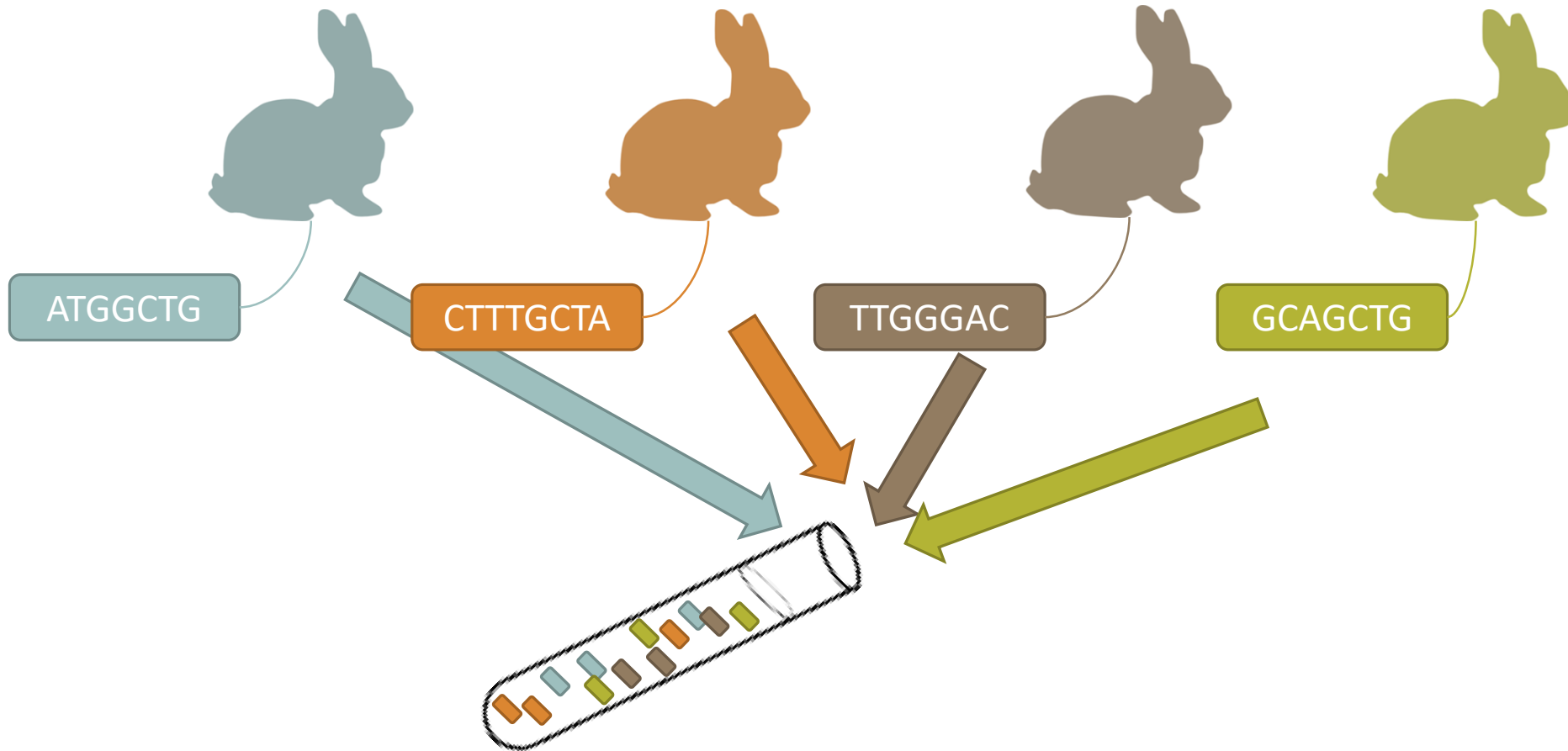
Farm animal feces
metagenome

`100spec_90000seq_9s
amples.tar.gz`

Demultiplexing tool



Barcoding ?



Demultiplexing

Sequence demultiplexing in function of barcode sequences :

- In forward
- In reverse
- In forward and reverse

Remove unbarcoded or ambiguous sequences

Demultiplexing forward



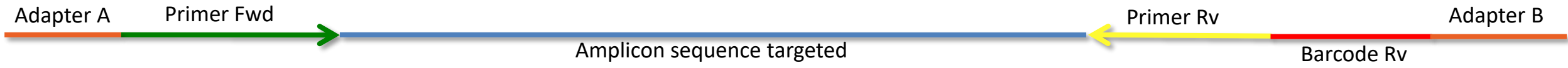
Single-end sequencing



Paired-end sequencing



Demultiplexing reverse



Single end sequencing



Paire end sequencing



Demultiplexing forward and reverse



Single end sequencing



Paired end sequencing



Your turn! - 1

LAUNCH DEMULTIPLEX READS TOOL

The tool parameters depend on the input data type

FROGS Demultiplex reads (version 1.1.0)

Barcode file:

This file describes barcodes and samples (one line by sample tabulated separated from barcode sequence(s)). See Help section

Single or Paired-end reads:

Select between paired and single end data

Select fastq dataset:

Specify dataset of your single end reads

barcode mismatches:

Number of mismatches allowed in barcode

barcode on which end ?:

at the beginning of the forward end or of the reverse end or both?

Forward
Reverse
Both ends
Execute

FROGS Demultiplex reads (version 1.1.0)

Barcode file:

This file describes barcodes and samples (one line by sample tabulated separated from barcode sequence(s)). See Help section

Single or Paired-end reads:

Select between paired and single end data

Select first set of reads:

Specify dataset of your forward reads

Select second set of reads:

Specify dataset of your reverse reads

barcode mismatches:

Number of mismatches allowed in barcode

barcode on which end ?:

at the beginning of the forward end or of the reverse end or both?

Forward
Reverse
Both ends
Execute

FROGS Demultiplex reads ✕

- Barcode file
- Select fastq dataset

demultiplexed_archive (data) 🗑

undemultiplexed_archive (data) 🗑

summary (tabular) 🗑

Exercise 1

In **multiplexed** history launch the demultiplex tool:

« The Patho-ID project, rodent and tick's pathobioms study, financed by the metaprogram INRA-MEM, studies zoonoses on rats and ticks from multiple places in the world, the co-infection systems and the interactions between pathogens. In this aim, they have extracted hundreds of or rats and ticks samples from which they have extracted 16S DNA and sequenced them first time on Roche 454 platform and in a second time on Illumina Miseq platform. For this courses, they authorized us to publicly shared some parts of these samples. »

Parasites & Vectors (2015) 8:172 DOI 10.1186/s13071-015-0784-7. **Detection of Orientia sp. DNA in rodents from Asia, West Africa and Europe.** Jean François Cosson, Maxime Galan, Emilie Bard, Maria Razzauti, Maria Bernard, Serge Morand, Carine Brouat, Ambroise Dalecky, Khalilou Bâ, Nathalie Charbonnel and Muriel Vayssier-Taussat

Exercise 1

In **multiplexed** history launch the demultiplex tool:

Data are **single end** reads




→ only 1 fastq file

Samples are characterized by one barcode in forward strands

→ multiplexing « **forward** »






Inputs :

```
2: /work/frogs          
/multiplex.fastq
```

```
1: /work/frogs          
/barcode forward.tabular
```


Exercise 1

Demultiplex tool asks for 2 files: one « fastq » and one « tabular »

1. Play with pictograms   
2. Observe how is built a fastq file. 
3. Look at the stdout, stderr when available (in the  pictogram)

Multiplex

FROGS Demultiplex reads Attribute reads to samples in function of inner barcode. (Galaxy Version 2.0.0) Options

Barcode file

24: barcode_forward.tabular

This file describes barcodes and samples (one line by sample tabulated separated from barcode sequence(s)). See Help section

Single or Paired-end reads

Single

Select between paired and single-end data

Select fastq dataset

6: multiplex.fastq

Specify dataset of your single end reads

Barcode mismatches

0

Number of mismatches allowed in barcode

Barcode on which end ?

Forward




The barcode is placed either at the beginning of the forward end or of the reverse end or both?




Advices




For your own data

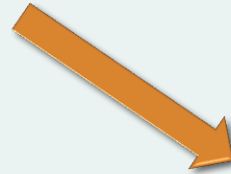
- Do not forget to indicate barcode sequence as they are in the fastq sequence file, especially if you have data multiplexed via the reverse strand.
- For the mismatch threshold, we advised you to let the threshold to 0, and if you are not satisfied by the result, try with 1. The number of mismatch depends on the length of the barcode, but often those sequences are very short so 1 mismatch is already more than the sequencing error rate.
- If you have different barcode lengths, you must demultiplex your data in different times beginning by the longest barcode set and used the "unmatched" or "ambiguous" sequence with smaller barcode and so on.
- If you have Roche 454 sequences in sff format, you must convert them with some program like [sff2fastq](#)

Results

9: FROGS Demultiplex   
reads: report

8: FROGS Demultiplex   
reads: undemultiplexed.tar.gz

7: FROGS Demultiplex   
reads: demultiplexed.tar.gz



1	2
#sample	count
ambiguous	0
MgArd0009	91
MgArd0017	166
MgArd0038	1208
MgArd0029	193
unmatched	245
MgArd0001	119
MgArd0081	246
MgArd0046	401
MgArd0054	243
MgArd0073	474
MgArd0062	1127

With barcode mismatches >1 sequence can corresponding to several samples. Sequence that match at only one sample are affected to this sample but the others (ambiguous) are not re-affected to a sample.

Sequences without known barcode. So these sequences are non-affected to a sample.

A tar archive is created by grouping one (or a pair of) fastq file per sample with the names indicated in the first column of the barcode tabular file

Format: Barcode

BARCODE FILE is expected to be **tabulated**:

- first column corresponds to the sample name (unique, without space)
- second to the forward sequence barcode used (None if only reverse barcode)
- optional third is the reverse sequence barcode (optional)

Take care to indicate sequence barcode in the strand of the read, so you may **need to reverse complement** the reverse barcode sequence. Barcode sequence must have the same length.

Example of barcode file.

The last column is optional, like this, it describes sample multiplexed by both fragment ends.

```
MgArd00001      ACAGCGT      ACGTACA
```

Format : FastQ

FASTQ : Text file describing biological sequence in 4 lines format:

- first line start by "@" correspond to the sequence identifier and optionally the sequence description. "@Sequence_1 description1"
- second line is the sequence itself. "ACAGC"
- third line is a "+" following by the sequence identifier or not depending on the version
- fourth line is the quality sequence, one code per base. The code depends on the version and the sequencer

```
@HNHOSKD01ALD0H  
ACAGCGTCAGAGGGGTACCAGTCAGCCATGACGTAGCACGTACA  
+  
CCCFHHHHHHJJJJHHFF@DEDDDDDDDD@CDDDDACDD
```

How it works ?

For each sequence or sequence pair the sequence fragment at the beginning (forward multiplexing) of the (first) read or at the end (reverse multiplexing) of the (second) read will be compared to all barcode sequences.

If this fragment is equal (with less or equal mismatch than the threshold) to one (and only one) barcode, the fragment is trimmed and the sequence will be attributed to the corresponding sample.

Finally fastq files (or pair of fastq files) for each sample are included in an archive, and a summary describes how many sequences are attributed for each sample.

Pre-process tool

FROGS Demultiplex reads ✕

- Barcode file
- Select fastq dataset

demultiplexed_archive (data)

undemultiplexed_archive (data)

summary (tabular)

Demultiplexing

Upload File from Genotoul ✕

out1 (bam, txt, tabular, fastqsanger, csfasta, qual, bed, gff, gtf, vcf, sam, fasta, pdf, xsq, tar.gz, bw, png, sff, pileup, pileupgz, zip)

Data acquisition



FROGS Pre-process ✕

- Archive file
- dereplicated_file (fasta)
- count_file (tabular)
- summary_file (html)

Pre-process

FROGS Clustering swarm ✕

- Sequences file
- Count file
- seed_file (fasta)
- abundance_biom (biom1)
- swarms_composition (tabular)

Clustering

FROGS Remove chimera ✕

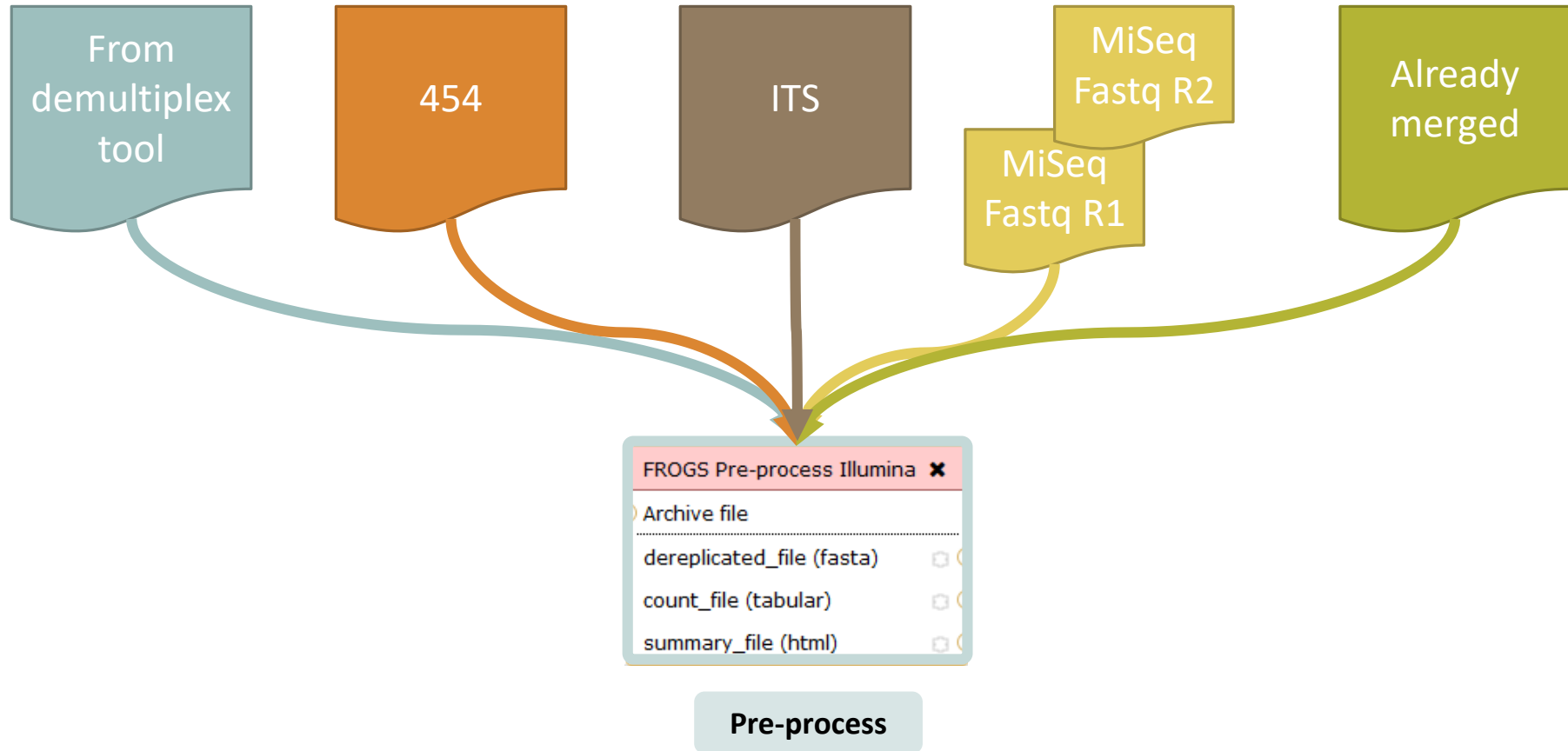
- Sequences file
- Abundance file
- non_chimera_fasta (fasta)
- out_abundance_biom (biom1)
- out_abundance_count (tabular)
- summary_file (html)

Chimera

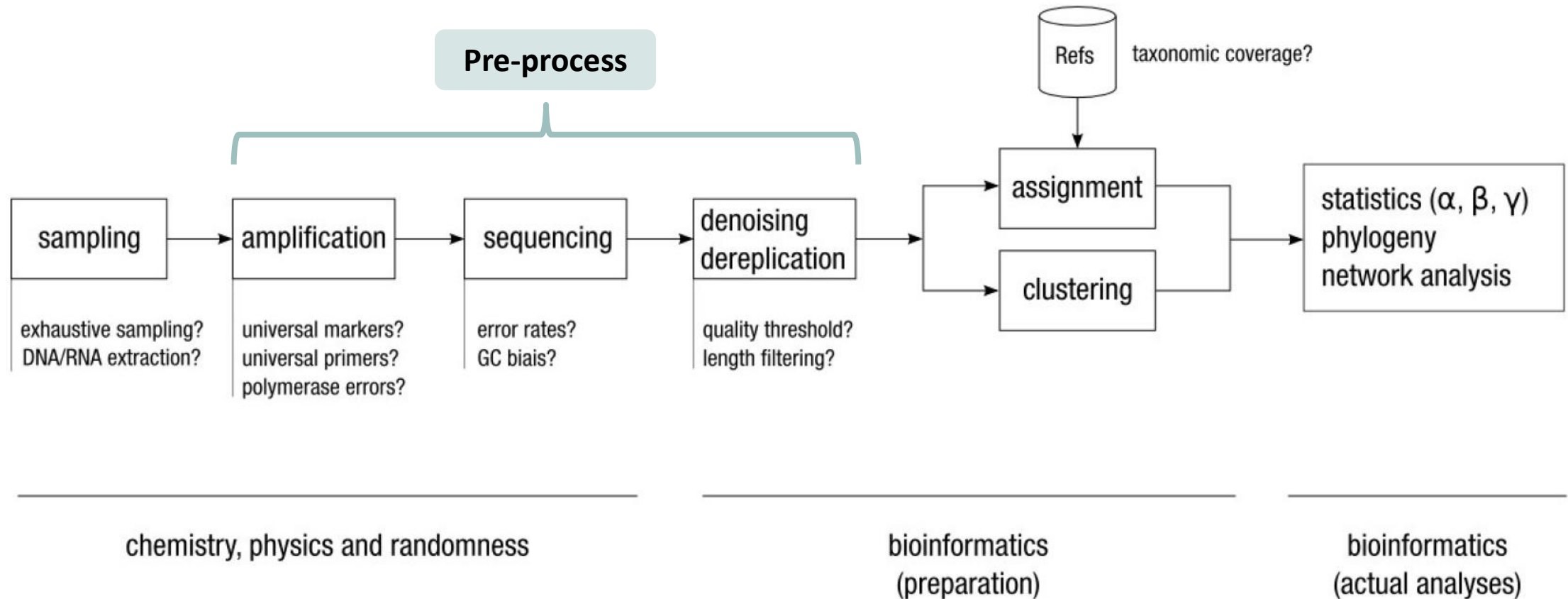
FROGS Affiliation OTU ✕

- OTU seed sequence
- Abundance file
- biom_affiliation (biom1)
- summary (html)

Affiliation



Amplicon-based studies general pipeline



Pre-process

- Delete sequence with not expected lengths
- Delete sequences with ambiguous bases (N)
- Delete sequences do not contain good primers
- Merging of reads
- Dereplication

- + removing homopolymers (size = 8) for 454 data
- + quality filter for 454 data

VSEARCH: a versatile open source tool for metagenomics.
Rognes T, Flouri T, Nichols B, Quince C, Mahé F.
PeerJ. 2016 Oct 18;4:e2584. eCollection 2016.

Example for:

- Illumina MiSeq data
- 1 sample
- Non joined

Pre-process example 1

FROGS Pre-process merging, denoising and dereplication. (Galaxy Version r3.0-3.0) Options

Sequencer
Illumina
Select the sequencing technology used to produce the sequences.

Input type
Files by samples
Samples files can be provided in single archive or with two files (R1 and R2) by sample.

Reads already contiged ?
No
The inputs contain 1 file by sample : R1 and R2 are already merged by pair.

Samples

1: Samples

Name
sampleA
The sample name.

Reads 1
1: http://genoweb.toulouse.inra.fr/~formation/15_FROGS/FROGS_ini/DATA/sampleA_R1.fastq
R1 FASTQ file of paired-end reads.

reads 2
2: http://genoweb.toulouse.inra.fr/~formation/15_FROGS/FROGS_ini/DATA/sampleA_R2.fastq
R2 FASTQ file of paired-end reads.

+ Insert Samples

Reads 1 size
250
The maximum read1 size.

Reads 2 size
250
The maximum read2 size.

mismatch rate.
0.1
The maximum rate of mismatches in the overlap

Merge software
Vsearch
Select the software to merge paired-end reads.

Would you like to keep unmerged reads?
Yes No
No : Unmerged reads will be excluded; Yes : unmerged reads will be artificially combined with 100 N. (default No)

Parameters for the merging

Minimum amplicon size

340

The minimum size for the amplicons.

Maximum amplicon size

450

The maximum size for the amplicons.

Sequencing protocol

Illumina standard

The protocol used for sequencing step: standard or custom with PCR primers as sequencing primers.

5' primer

CCGTCAATTC

The 5' primer sequence (wildcards are accepted). The orientation is detailed below in 'Primers parameters'.

3' primer

CCGCNGCTGCT

The 3' primer sequence (wildcards are accepted). The orientation is detailed below in 'Primers parameters'.

✓ Execute

[V5] 16S variability

Primer sequences

Pre-process example 1

Example for:

- Sanger 454 data
- 1 sample
- Only one read (454 process)

FROGS Pre-process Step 1 in metagenomics analysis: denoising and dereplication. (Galaxy Version 1.5.0) Options

Sequencer
454
Select the sequencer family used to produce the sequences.

Input type
One file by sample
Samples files can be provided in single archive or with one file by sample.

Samples
1: Samples

Name
my_sample
The sample name.

Sequence file
1: /work/formation/FROGS/454.fastq.gz
FASTQ file of sample.

Minimum amplicon size
380
The minimum size for the amplicons (with primers).

Maximum amplicon size
500
The maximum size for the amplicons (with primers).

5' primer
ACGGGAGGCAGCAG
The 5' primer sequence (wildcards are accepted). The orientation is detailed below in 'Primers parameters'.

3' primer
AGGATTAGATACCCTGGTA
The 3' primer sequence (wildcards are accepted). The orientation is detailed below in 'Primers parameters'.

Execute

[V3 – V4] 16S variability

Primer sequences

Pre-process example 2

Example for:

- Illumina MiSeq data
- 9 samples in 1 archive
- Joined
- Without sequenced PCR primers (Kozich protocol)

FROGS Pre-process Step 1 in metagenomics analysis: denoising and dereplication. (Galaxy Version 1.5.0) Options

Sequencer
Illumina **Sequencing technology**
Select the sequencer family used to produce the sequences.

Input type
Archive **One file per sample and all files are contained in a archive**
Samples files can be provided in single archive or with two files (R1 and R2) by sample.

Archive file
1: /work/project/frogs/Formation/100spec_90000seq_9samples_Hantagulumic.tar.gz
The tar file containing the sequences file(s) for each sample.

Reads already contiged ?
Yes **Paire-end sequencing all ready joined**
The archive contains 1 file by sample : Reads 1 and Reads 2 are already contiged by pair.

Minimum amplicon size
380 **[V3 – V4] 16S variability**
The minimum size for the amplicons.

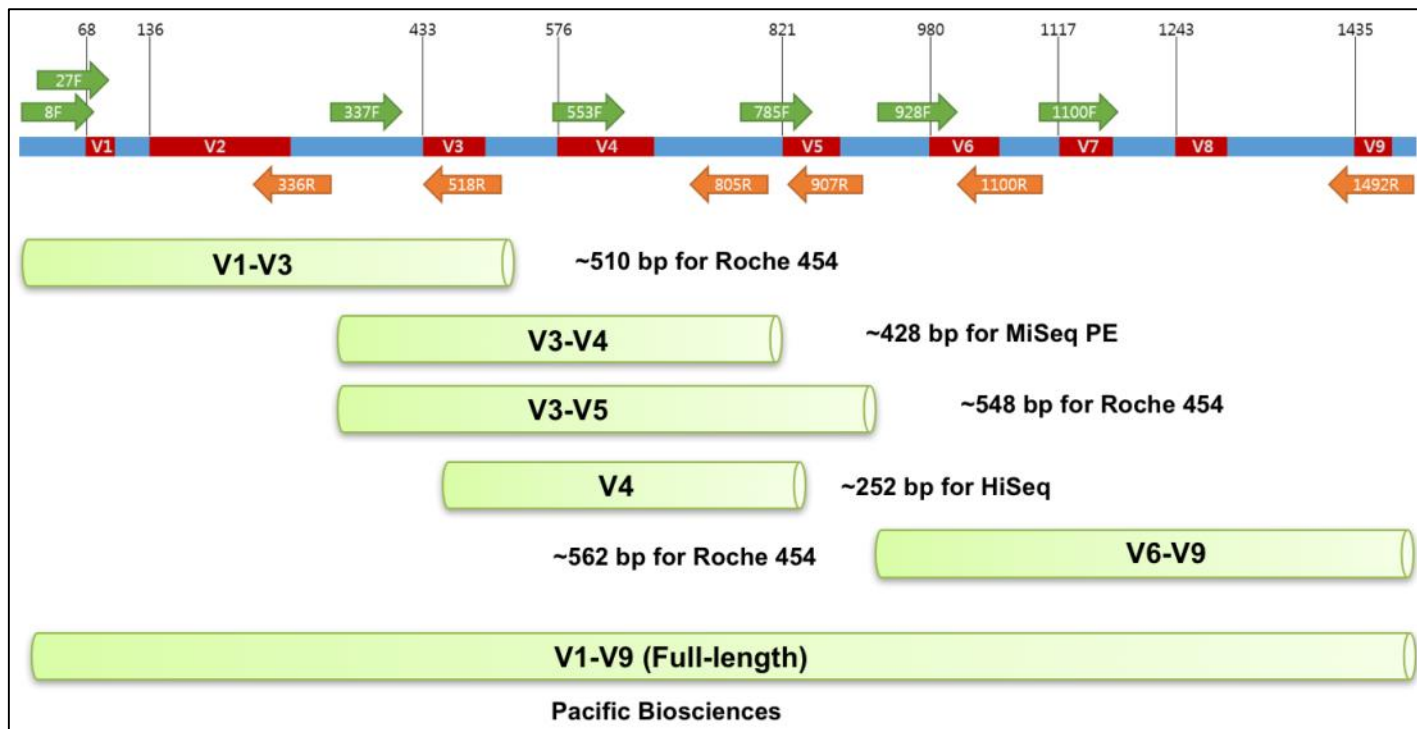
Maximum amplicon size
500
The maximum size for the amplicons.

Sequencing protocol
Custom protocol (Kozich et al. 2013) **No more primers**
The protocol used for sequencing step: standard or custom with PCR primers as sequencing primers.

Execute

Pre-process example 3

Which primers for 16S ?



Name of primer F=forward, R=reverse	Sequence
8F	AGAGTTTGATCCTGGCTCAG
27F	AGAGTTTGATCMTGGCTCAG
336R	ACTGCTGCSYCCCGTAGGAGTCT
337F	GACTCCTACGGGAGGCWGCAG
337F	GACTCCTACGGGAGGCWGCAG
341F	CCTACGGGNGGCWGCAG
515FB	GTGYCAGCMGCCGCGGTAA
518R	GTATTACCGGGCTGCTGG
533F	GTGCCAGCMGCCGCGGTAA
785F	GGATTAGATACCCTGGTA
805R	GACTACHVGGGTATCTAATCC
806RB	GGACTACNVGGGTWTCTAAT
907R	CCGTCAATTCCTTTRAGTTT
928F	TAAACTYAAAKGAATTGACGGG
1100F	YAACGAGCGCAACCC
1100R	GGGTTGCGCTCGTTG
1492R	CGGTTACCTTGTACGACTT

NGS platforms	16S region	PCR primers	Estimated insert size to read (E. coli)	Sequencing
Illumina MiSeq PE (Pair End)	V3V4	341F & 805R	427 bp	250 bp x 2 or 300 bp x 2
Illumina HiSeq/iSeq100 (Earth Microbiome Project)	V4	515FB & 806RB	250 bp	150 x 2

Your turn! - 2

Exercise 2.1

Go to « 454 » history

Launch the pre-process tool on that data set

→ objective : understand the parameters

1- Test different parameters for « minimum and maximum amplicon size »

2- Enter these primers: Forward: ACGGGAGGCAGCAG Reverse: AGGATTAGATACCCTGGTA

454

FROGS Pre-process merging, denoising and dereplication. (Galaxy Version r3.0-3.0) Options

Sequencer
454
Select the sequencing technology used to produce the sequences.

Input type
One file by sample
Samples files can be provided in single archive or with one file by sample.

Samples

1: Samples

Name
my_sample
The sample name.

Sequence file
1: http://genoweb.toulouse.inra.fr/~formation/15_FROGS/FROGS_ini/DATA/454.fastq
FASTQ file of sample.

Minimum amplicon size
380
The minimum size for the amplicons (with primers).

Maximum amplicon size
500
The maximum size for the amplicons (with primers).

5' primer
ACGGGAGGCAGCAG
The 5' primer sequence (wildcards are accepted). The orientation is detailed below.

3' primer
AGGATTAGATACCCTGGTA
The 3' primer sequence (wildcards are accepted). The orientation is detailed below.

Execute

Sample name is required

Size range of 16S V3-V4:
[380 – 500]

Primers used for sequencing V3-V4:
Forward: ACGGGAGGCAGCAG
Reverse: AGGATTAGATACCCTGGTA

Exercise 2.1

What do you understand about amplicon size, which file can help you ?

What is the length of your reads before preprocessing ?

Do you understand how enter your primers ?



What is the « FROGS Pre-process: dereplicated.fasta » file ?



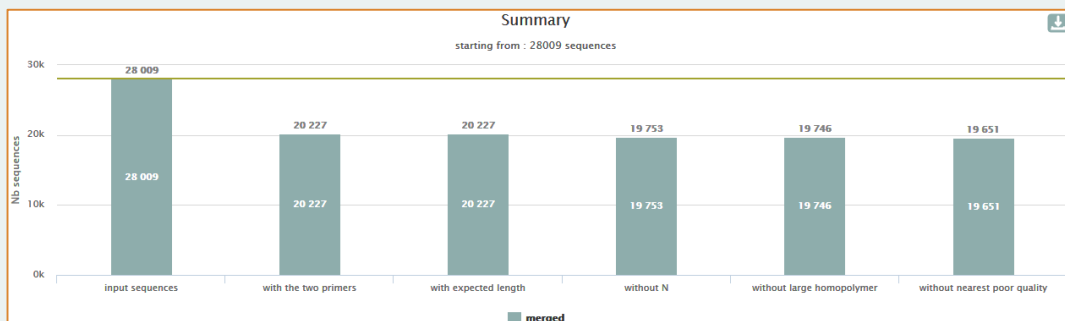
What is the « FROGS Pre-process: count.tsv » file ?



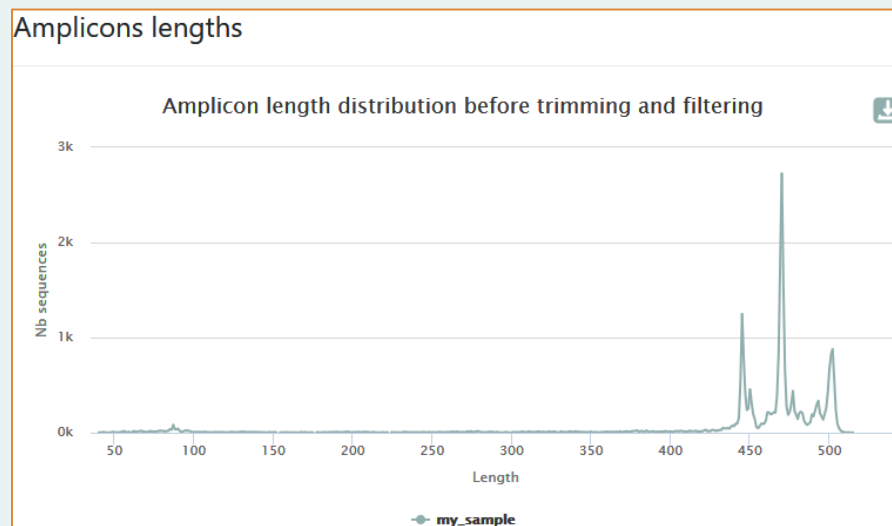
Explore the file « FROGS Pre-process: report.html »

Who loose a lot of sequences ?

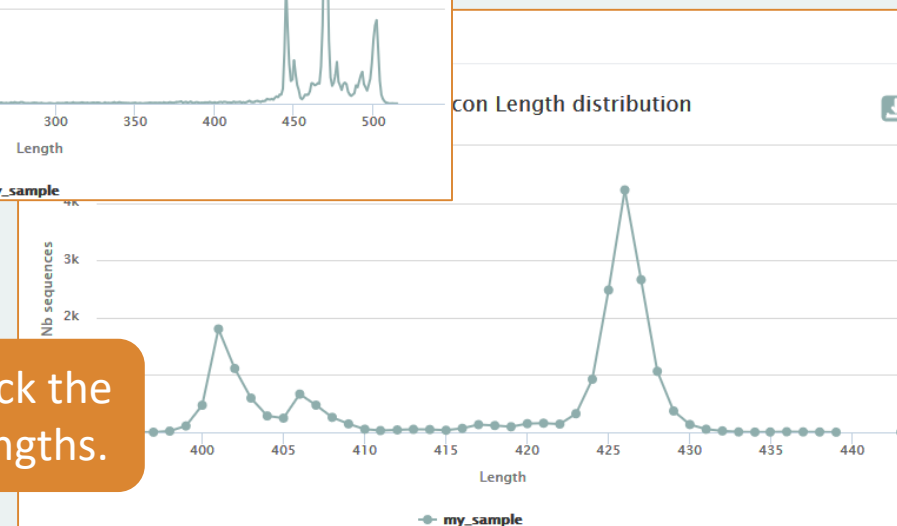
<input checked="" type="checkbox"/>	Samples \updownarrow	% kept \updownarrow	input sequences \updownarrow	with the two primers \updownarrow	with expected length \updownarrow	without N \updownarrow	without large homopolymer \updownarrow	without nearest poor quality \updownarrow
<input checked="" type="checkbox"/>	my_sample	70.16	28,009	20,227	20,227	19,753	19,746	19,651



To be kept, sequences must have the 2 primers



To adjust your filtering, check the distribution of sequence lengths.



Cleaning, how it work ?

Filter contig sequence **on its length** which must be between min-amplicon-size and max-amplicon-size

use **cutadapt** to search and **trim primers** sequences with less than 10% differences

Minimum amplicon size:

The minimum size for the amplicons.

Maximum amplicon size:


The maximum size for the amplicons.

Cleaning, how it work ?

dereplicate sequences and return one **uniq fasta file** for all sample and a **count table** to indicate **sequence abundances among sample**.

In the HTML report file, you will find for each filter the number of sequences passing it, and a table that details these filters for each sample.

Pre-process

- Delete sequence with not expected lengths
- Delete sequences with ambiguous bases (N)
- Delete sequences do not contain good primers
- Merging of reads 
- Dereplication

- + removing homopolymers (size = 8) for 454 data
- + quality filter for 454 data

VSEARCH: a versatile open source tool for metagenomics.
Rognes T, Flouri T, Nichols B, Quince C, Mahé F.
PeerJ. 2016 Oct 18;4:e2584. eCollection 2016.

The aim of Vsearch is to merge R1 with R2

Case of a sequencing of overlapping sequences: case of 16S V3-V4 amplicon MiSeq sequencing:

Imagine a real amplicon sequence of 400bp

400bp



Imagine a Miseq paired sequencing of 2x250bp

R1 : 250bp



R2 : 250bp



Reconstructing amplicon sequence is possible thanks to the overlap region



Merged sequence length : 400bp, with 100bp overlap

The aim of Vsearch is to merge R1 with R2

Case of a sequencing of over-overlapping sequences:

Imagine a real amplicon sequence of 200bp

200bp



Imagine a Miseq paired sequencing of 2x250bp

R1 : 250bp



R2 : 250bp



FROGS takes in charge this case in trimming over bases

200bp



Merged sequence length : 200bp, with 100% overlap

Exercise 2.2

Go to « [MiSeq R1 R2](#) » history

Launch the pre-process tool on that data set

→ objective: understand Vsearch software

Miseq R1
R2

```
>ERR619083.M00704
CCGTCAATTCATTGAGTTTCAACCTTGC GGCCG TACTTCCCAGGCGGTACGTT
TATCGCGTTAGCTTCGCCAAGCACAGCATCCTGCGCTTAGCCAACGTACATCG
TTTAGGGTGTGGACTACCCGGGTATCTAATCCTGTTTCGCTACCCACGCTTTCG
AGCCTCAGCGTCAGTGACAGACCAGAGACCGCTTTCGCCACTGGTGTTCCTC
CATATATCTACGCATTTACCCGCTACACATGGAATTCCACTCTCCCCTTCTGC
ACTCAAGTCAGACAGTTTCCAGAGCACTCTATGGTTGAGCCATAGCCTTTTAC
TCCAGACTTTCCTGACCGACTGCACTCGCTTTACGCCAATAAATCCGGACAA
CGCTTGCCACCTACGTATTA CCGCNGCTGCT
```

Real 16S sequenced
fragment

Sequencer
Illumina
Select the sequencing technology used to produce the sequences.

Input type
Files by samples
Samples files can be provided in single archive or with two files (R1 and R2) by sample.

Reads already contiged ?
No
The inputs contain 1 file by sample. R1 and R2 are already merged by pair.

Samples

1: Samples

Name
sampleA
The sample name.

Reads 1
59: /work/formation/FROGS/sampleA_R1.fastq
R1 FASTQ file of paired-end reads.

reads 2
60: /work/formation/FROGS/sampleA_R2.fastq
R2 FASTQ file of paired-end reads.

+ Insert Samples

Reads 1 size
250
The read1 size.

Reads 2 size
250
The read2 size.

Sample name is required

mismatch rate.
0.1
The maximum rate of mismatches in the overlap region

Merge software
Vsearch
Select the software to merge paired-end reads.

Would you like to keep unmerged reads?
Yes No
No : Unmerged reads will be excluded; Yes : unmerged reads will be artificially combined with 100 N. (default No)

Minimum amplicon size
340
The minimum size for the amplicons (with primers).

Maximum amplicon size
450
The maximum size for the amplicons (with primers).

Sequencing protocol
Illumina standard
The protocol used for sequencing

5' primer
CCGTCAATTC
The 5' primer sequence (wildcards are accepted).

3' primer
CCGCNGCTGCT
The 3' primer sequence (wildcards are accepted). The orientation is detailed below in 'Primers parameters'.

Do not use flash

Reads can be
overlapped

Primers used for sequencing V5 region:
Forward: CCGTCAATTC
Reverse: CCGCNGCTGCT
Lecture 5' → 3'

Execute

Exercise 2.2

What do you understand about amplicon size, which file can help you ?

What is the length of your reads before preprocessing ?

Do you understand how enter your primers ?



What is the « FROGS Pre-process: dereplicated.fasta » file ?



What is the « FROGS Pre-process: count.tsv » file ?



Explore the file « FROGS Pre-process: report.html »

Who loose a lot of sequences ?

Expected amplicon size

410

Maximum amplicon length expected in approximately 90% of the amplicons.

mismatch rate.

0.1

The maximum rate of mismatches in the overlap region

Minimum amplicon size

340

The minimum size for the amplicons.

Maximum amplicon size

450

The maximum size for the amplicons.

Sequencing protocol

illumina standard

The protocol used for sequencing step: standard or custom with PCR primers as sequencing primers.

5' primer

CCGTCAATTC

The 5' primer sequence (wildcards are accepted). The orientation is detailed below in 'Primers parameters'.

3' primer

CCGCNGCTGCT

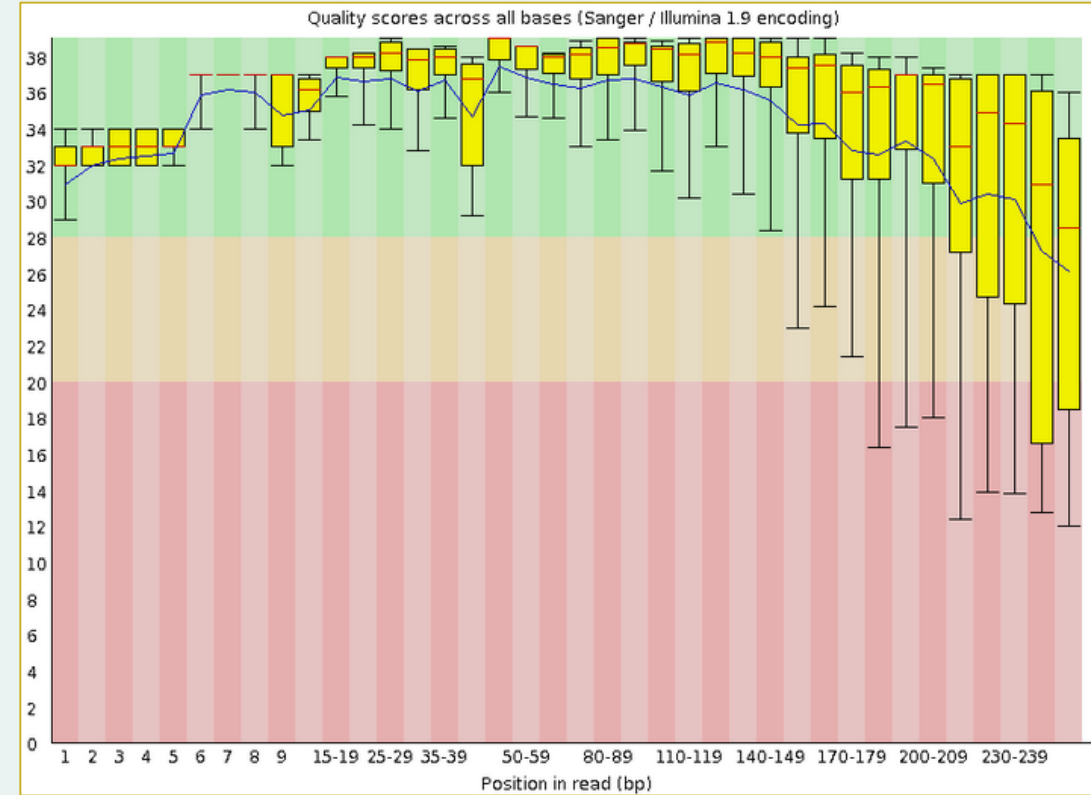
The 3' primer sequence (wildcards are accepted). The orientation is detailed below in 'Primers parameters'.

Execute

To increase, if your sequences have low qualities
Use FASTQC to know it!

FastQC: fastq/sam/bam

FastQC:Read QC reports using FastQC



Exercise 2.3

Go to « ITS » history

Launch the pre-process tool on this data set

→ objective : understand the « combined sequences »

→ objective : work with non-overlapping reads

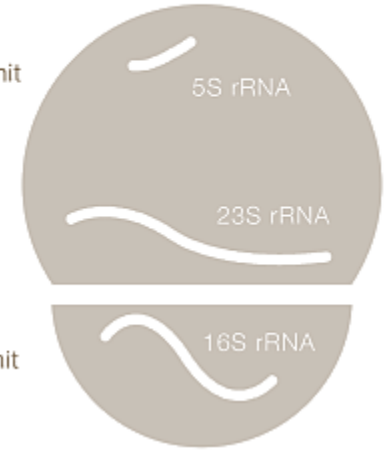
1- Enter these primers:

Forward: CTTGGTCATTTAGAGGAAGTAA Reverse: GCATCGATGAAGAACGCAGC

What is a ITS ?

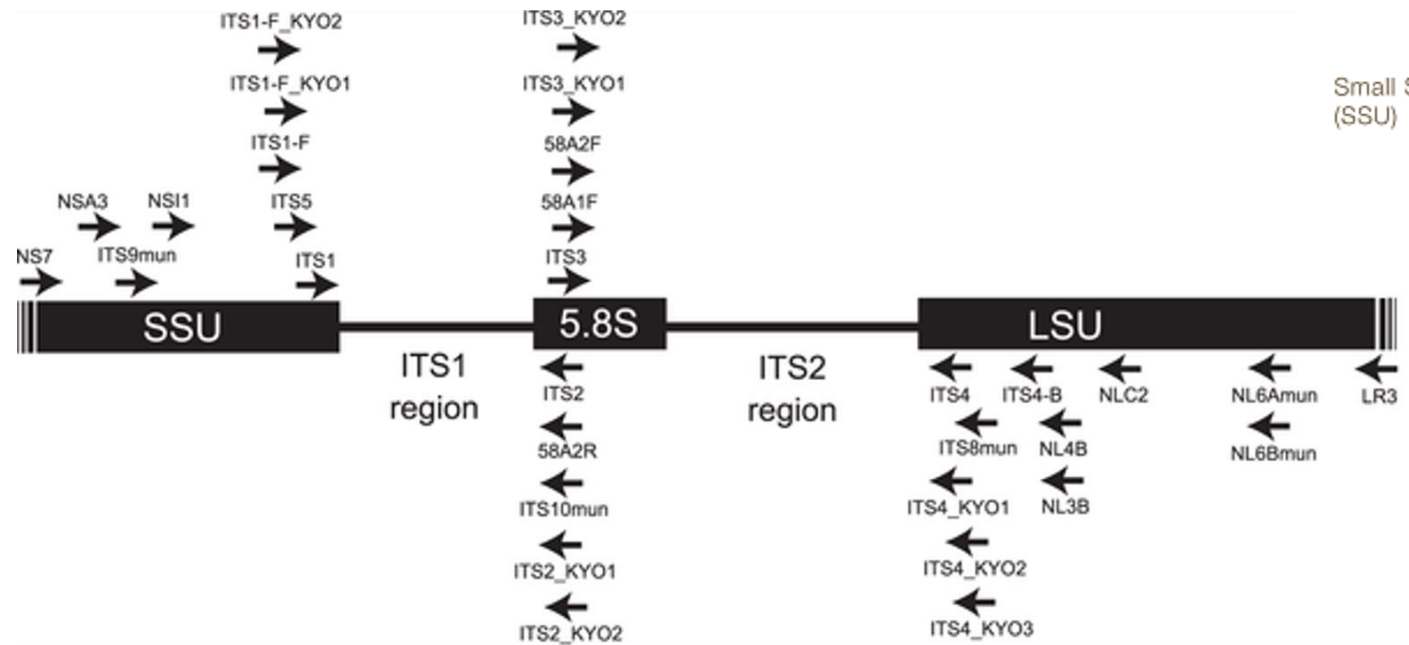
Prokaryotic Ribosome

Large Subunit (LSU)



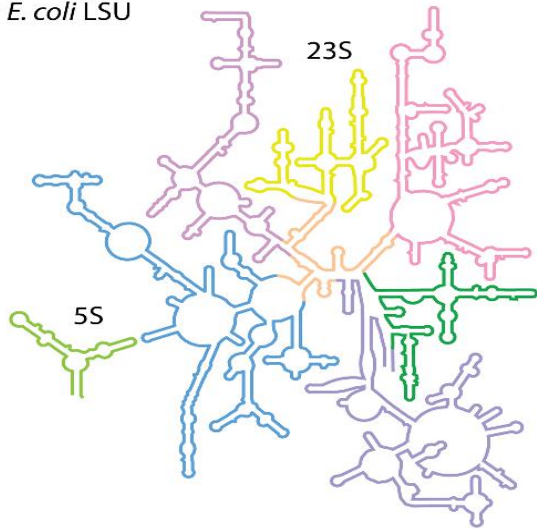
Small Subunit (SSU)

Map of nuclear ribosomal RNA genes and their ITS regions.

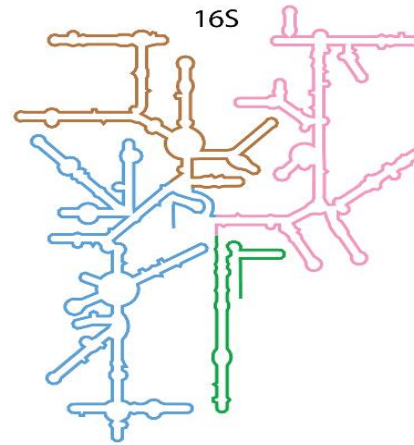


Toju H, Tanabe AS, Yamamoto S, Sato H (2012) High-Coverage ITS Primers for the DNA-Based Identification of Ascomycetes and Basidiomycetes in Environmental Samples. PLOS ONE 7(7): e40863. <https://doi.org/10.1371/journal.pone.0040863>

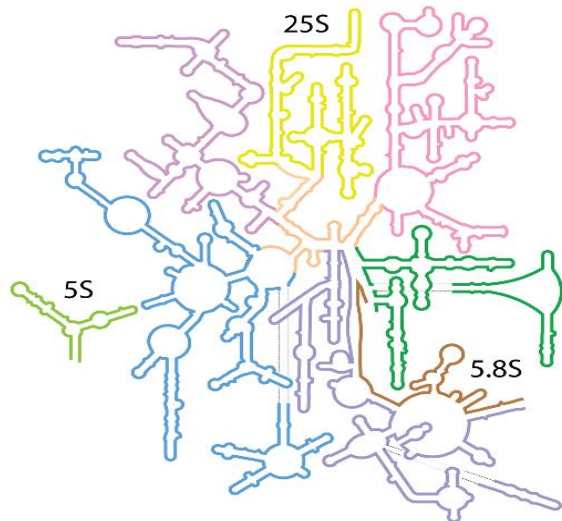
a) *E. coli* LSU



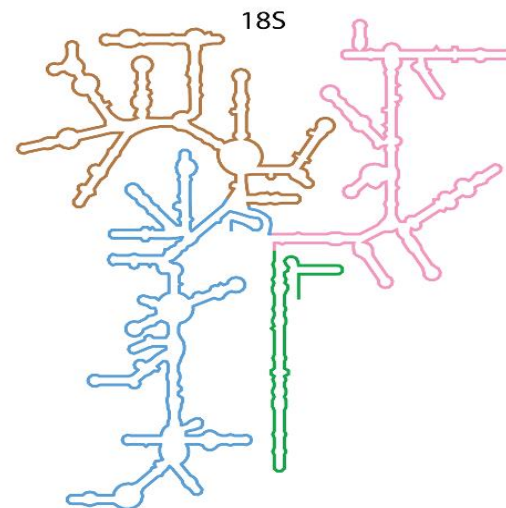
b) *E. coli* SSU



c) *S. cerevisiae* LSU



d) *S. cerevisiae* SSU



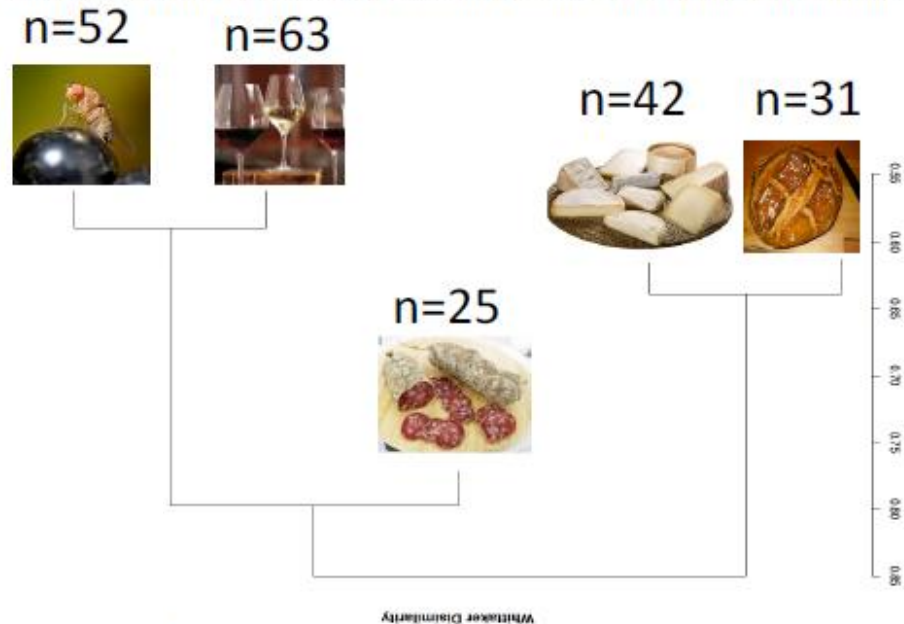
Schematic rRNA 2° structures of a) *E. coli* LSU, b) *E. coli* SSU, c) *S. cerevisiae* LSU, and d) *S. cerevisiae* SSU. These 2° structures are derived from 3D structures, and include non-canonical base pairs.

Secondary Structures of rRNAs from All Three Domains of Life
Anton S. Petrov, Chad R. Bernier, Burak Gulen, Chris C. Waterbury,
Eli Hershkovits, Chiaolong Hsiao, Stephen C. Harvey, Nicholas V. Hud,
George E. Fox, Roger M. Wartell, Loren Dean Williams
February 5, 2014 <https://doi.org/10.1371/journal.pone.0088222>

ITS data form METABARFOOD Project metaprogramme MEM

Yeast catalog in food ecosystems

Number of yeast species reported at least twice in each ecosystem
and their dissimilarity between ecosystems, as measured by the Whittaker distance



- While metabarcoding is commonly used to describe prokaryotes in the microbiome of many environments, methods for describing micro-eukaryote diversity is lacking and requires better methodology and standardisation.
- One reason is that the universal fungal barcode, the Internal Transcribed Spacer (ITS) region, displays considerable size variation amongst yeasts and other micro-eukaryotes.
- There are also several repeats leading to sequencing errors or termination.
- Additionally, the ITS databases are far from complete, especially for Ascomycota that are commonly found in food.
- Other rDNA barcodes have been used but often do not harbor enough polymorphism to detect taxa to the species level.
- In food, microbiota are usually composed of a reduced number of species compared to wild environments.
- Detecting micro-eukaryotes at the species level, and potentially strain level, is therefore necessary.

Sequencer

Illumina

Select the sequencing technology used to produce the sequences.

Input type

Archive

Samples files can be provided in single archive or with two files (R1 and R2) by sample.

Archive file

1: /work/frogsfungi/ITS.tar.gz

The tar file containing the sequences file(s) for each sample.

Reads already merged ?

No

The archive contains 1 file by sample : R1 and R2 are already merged by pair.

Reads 1 size

250

The maximum read1 size.

Reads 2 size

250

The maximum read2 size.

mismatch rate.

0.1

The maximum rate of mismatch in the overlap region

Merge software

Vsearch

Select the software to merge paired-end reads.

Would you like to keep unmerged reads? Yes No

No : Unmerged reads will be excluded; Yes : unmerged reads will be artificially combined with 100 N. (default No)

To keep FROGS combined sequences, choose YES

Minimum amplicon size

The minimum size for the amplicons (with primers).

Maximum amplicon size

The maximum size for the amplicons (with primers).

Sequencing protocol

The protocol used for sequencing step: standard or custom with PCR primers as sequencing primers.

5' primer

The 5' primer sequence (wildcards are accepted). The orientation is detailed below in 'Primers parameters'.

3' primer

The 3' primer sequence (wildcards are accepted). The orientation is detailed below in 'Primers parameters'.

Exercise 2.3

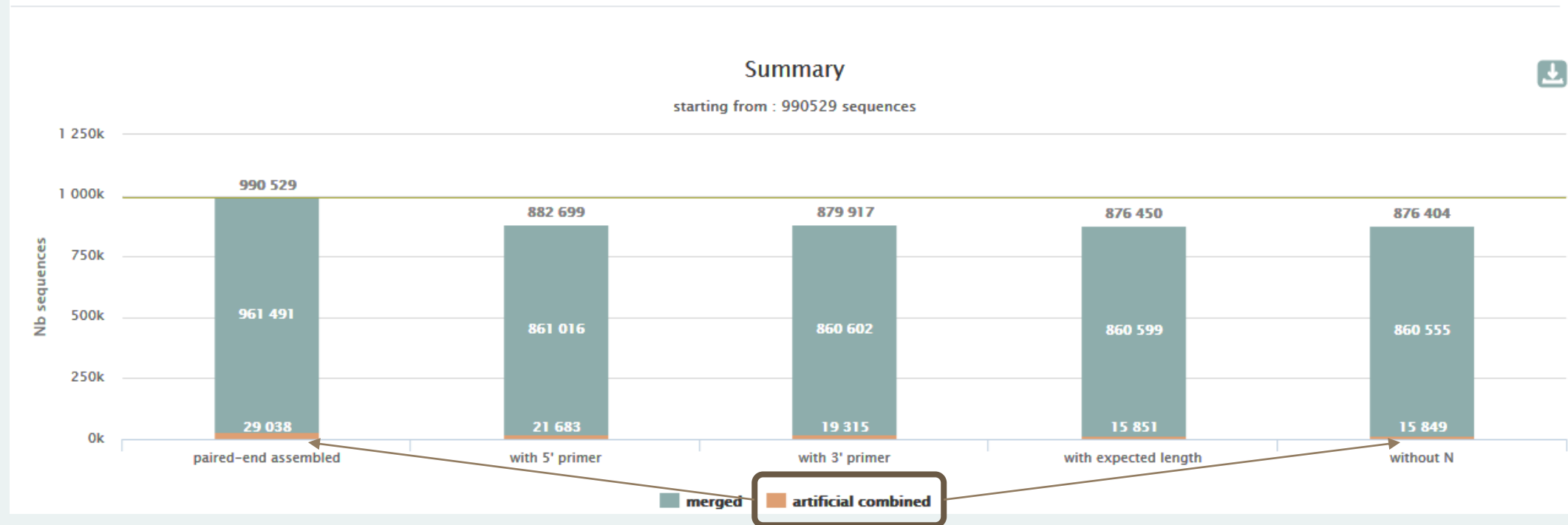
Go to « **ITS** » history

Launch the pre-process tool on this data set

→ objective: understand preprocess report and « FROGS combined sequences »

Explore Preprocess report.html

Preprocess summary



Explore Preprocess report.html

2 tables:

Details on merged sequences

Show entries Search: [CSV](#)

<input type="checkbox"/>	Samples	% kept	paired-end assembled	with 5' primer	with 3' primer	with expected length	without N
<input type="checkbox"/>	complexe-ADN-1	91.09	54,121	49,322	49,303	49,303	49,299
<input type="checkbox"/>	echantillon1-1	84.93	31,836	27,059	27,040	27,040	27,039
<input type="checkbox"/>	echantillon1-2	94.73	54,774	51,938	51,895	51,895	51,890
<input type="checkbox"/>	echantillon1-3	74.90	81,611	61,197	61,135	61,134	61,128
<input type="checkbox"/>	echantillon2-1	90.17	51,984	46,886	46,875	46,874	46,873

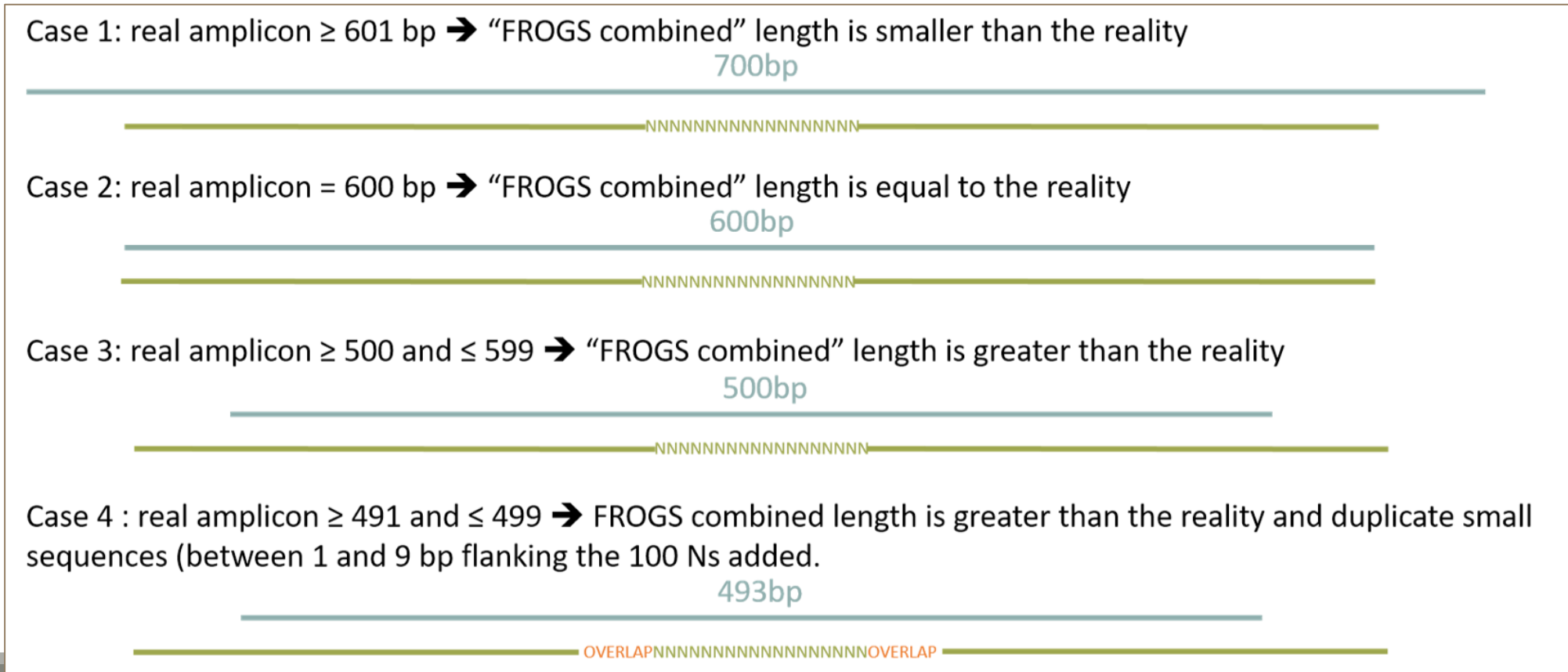
Details on **artificial combined sequences**

Show entries Search: [CSV](#)

<input type="checkbox"/>	Samples	% kept	paired-end assembled	with 5' primer	with 3' primer	with expected length	without N
<input type="checkbox"/>	complexe-ADN-1	68.47	2,163	1,833	1,656	1,481	1,481
<input type="checkbox"/>	echantillon1-1	54.92	1,047	751	620	575	575
<input type="checkbox"/>	echantillon1-2	61.57	1,392	1,096	942	858	857
<input type="checkbox"/>	echantillon1-3	49.54	2,491	1,617	1,334	1,234	1,234
<input type="checkbox"/>	echantillon2-1	44.62	1,421	996	899	634	634

FROGS "combined" sequences are artificial and present particular features especially on size.

Imagine a MiSeq sequencing of 2x250pb with reads impossible to overlap. So FROGS "combined" length = 600 bp.



Exercise 2.4

Go to« [MiSeq merged](#) » history

Launch the pre-process tool on that data set

→ objective: understand output files

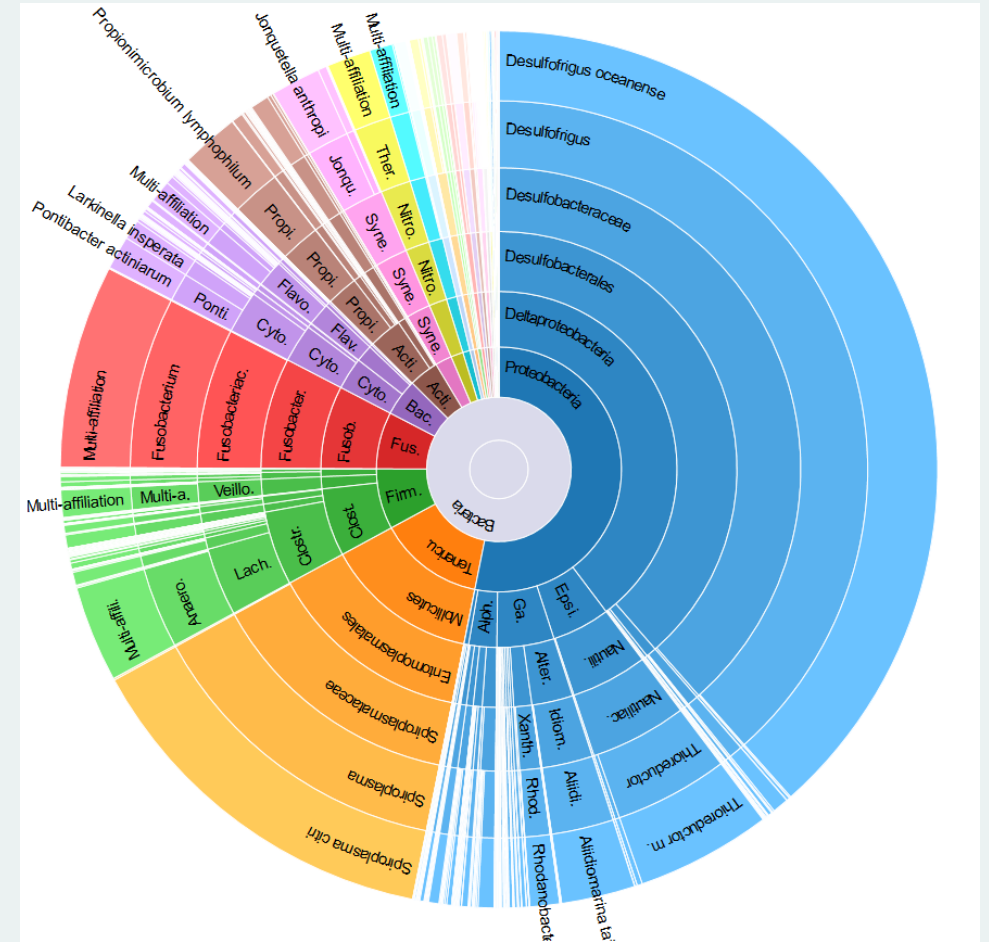
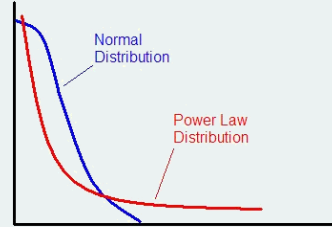
Exercise 2.4

3 samples are **technically replicated** 3 times : 9 samples of 10 000 sequences each.

100_10000seq_sampleA1.fastq	100_10000seq_sampleB1.fastq	100_10000seq_sampleC1.fastq
100_10000seq_sampleA2.fastq	100_10000seq_sampleB2.fastq	100_10000seq_sampleC2.fastq
100_10000seq_sampleA3.fastq	100_10000seq_sampleB3.fastq	100_10000seq_sampleC3.fastq

Exercise 2.4

- 100 species, covering all bacterial phyla
- Power Law distribution of the species abundances
- Error rate calibrated with real sequencing runs
- 10% chimeras
- 9 samples of 10 000 sequences each (90 000 sequences)



Exercise 2.4

“Grinder (v 0.5.3) (Angly et al., 2012) was used to simulate the PCR amplification of full-length (V3-V4) sequences from reference databases. The reference database of size 100 were generated from the LTP SSU bank (version 115) (Yarza et al., 2008) by

- (1) filtering out sequences with a N,
- (2) keeping only type species
- (3) with a match for the forward (ACGGGAGGCAGCAG) and reverse (TACCAGGGTATCTAATCCTA) primers in the V3-V4 region and
- (4) maximizing the phylogenetic diversity (PD) for a given database size. The PD was computed from the NJ tree distributed with the LTP.”

FROGS Pre-process Step 1 in metagenomics analysis: denoising and dereplication. (Galaxy Version 2.0.0) Options

Sequencer

▼

Select the sequencing technology used to produce the sequences.

Input type

▼

Samples files can be provided in single archive or with two files (R1 and R2) by sample.

Archive file

▼

The tar file containing the sequences file(s) for each sample.

Reads already contiged ?

▼

The archive contains 1 file by sample : R1 and R2 are already merged by pair.

Minimum amplicon size

The minimum size for the amplicons.

Maximum amplicon size

The maximum size for the amplicons.

Sequencing protocol

▼

The protocol used for sequencing step: standard or custom with PCR primers as sequencing primers.

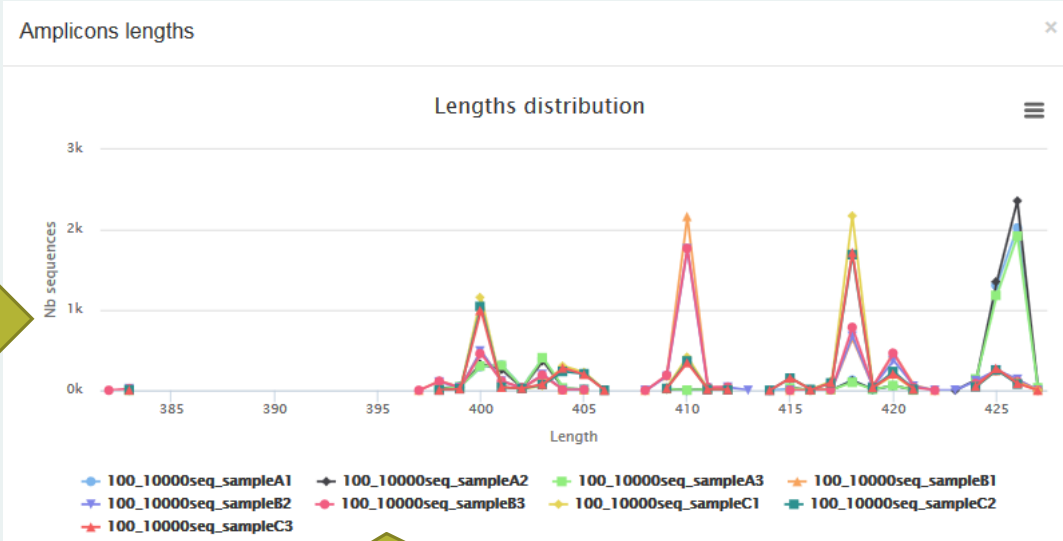
5' primer

The 5' primer sequence (wildcards are accepted). The orientation is detailed in the primer list.

3' primer

The 3' primer sequence (wildcards are accepted). The orientation is detailed in the primer list.

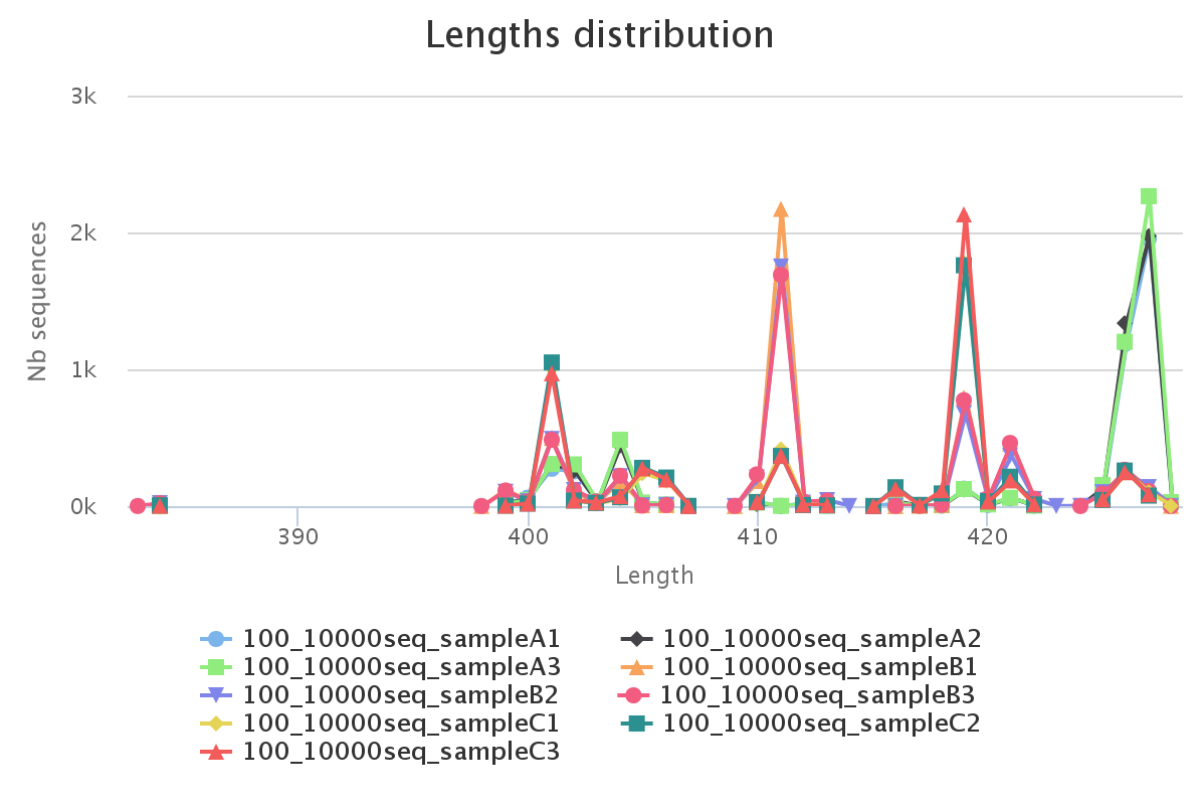
Execute



Click on legend

Primers used for this sequencing :
 5' primer: ACGGGAGGCAGCAG
 3' primer: TAGGATTAGATACCCTGGTA
 Lecture 5' → 3'

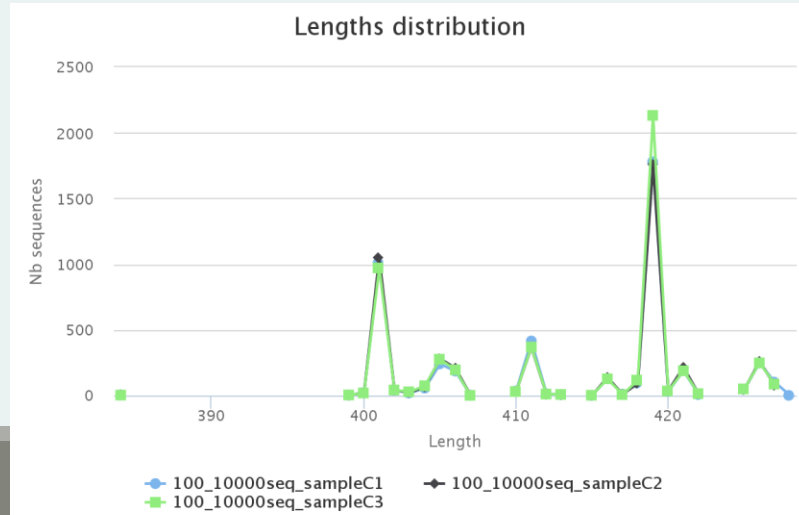
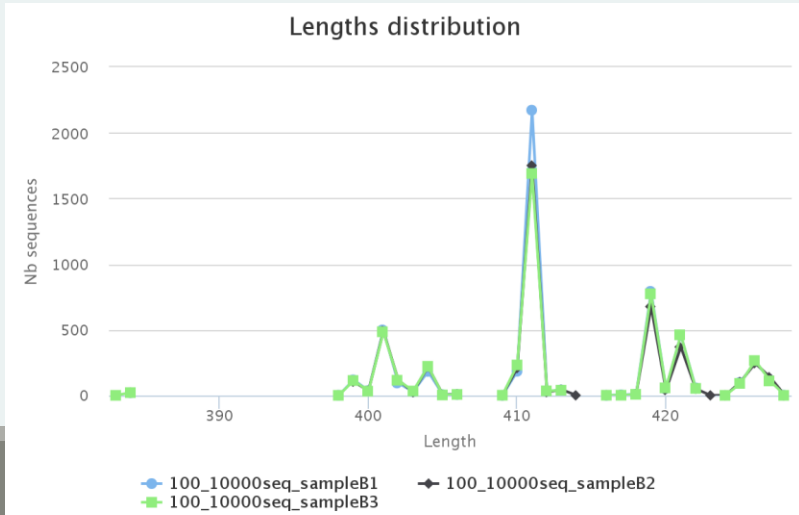
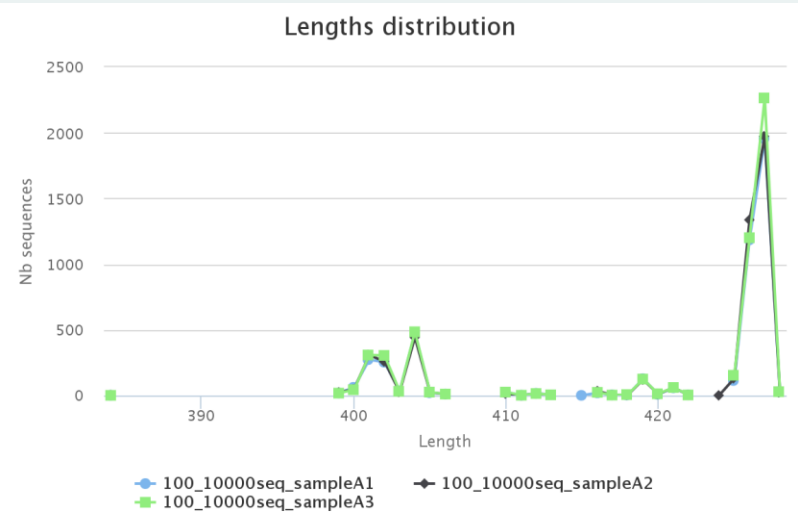
Miseq merged



Samples A only

Samples B only

Samples C only



Exercise 2.4 - Questions

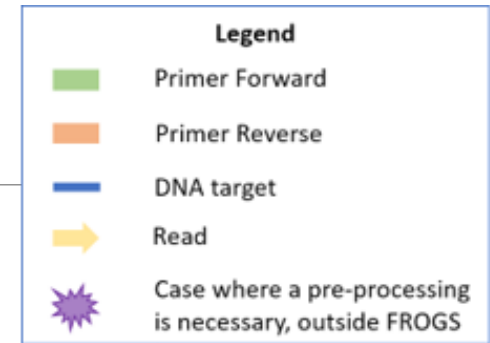
1. How many sequences are there in the input file ?
2. How many sequences did not have the 5' primer?
3. How many sequences still are after pre-processing the data?
4. How much time did it take to pre-process the data ?
5. What can you tell about the sample based on sequence length distributions ?

Preprocess tool in bref

	Take in charge
Illumina	✓
454	✓
Merged data	✓
Not merged data	✓
Without primers	✓
Only R1 or only R2	⊘
Too distant R1 and R2 to be merged	✓
Over-overlapping R1 R2	✓

	Take in charge
Archive .tar.gz	✓
Fastq	✓
Fasta	⊘
With only 1 primer	⊘
Multiplexed data	⊘
Demultiplexed data	✓

Processed data by FROGS in bref



454



illumina

Standard sequencing protocol

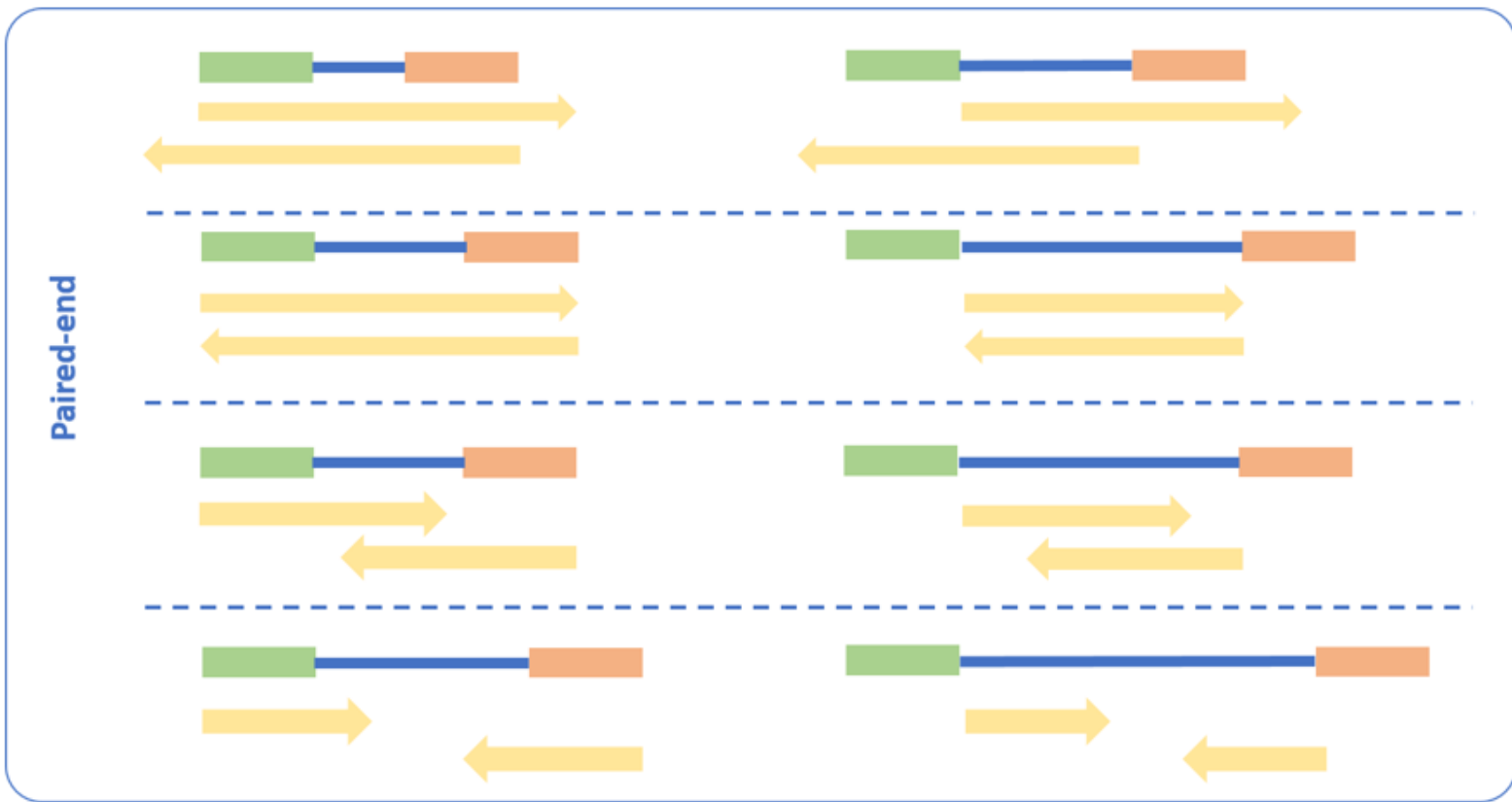
Kozich protocol : primers are not included in reads



→ Remove reverse primer before FROGS processing

Legend

- Primer Forward
- Primer Reverse
- DNA target
- Read
- Case where a pre-processing is necessary, outside FROGS



Length of the sequenced target < length of one read

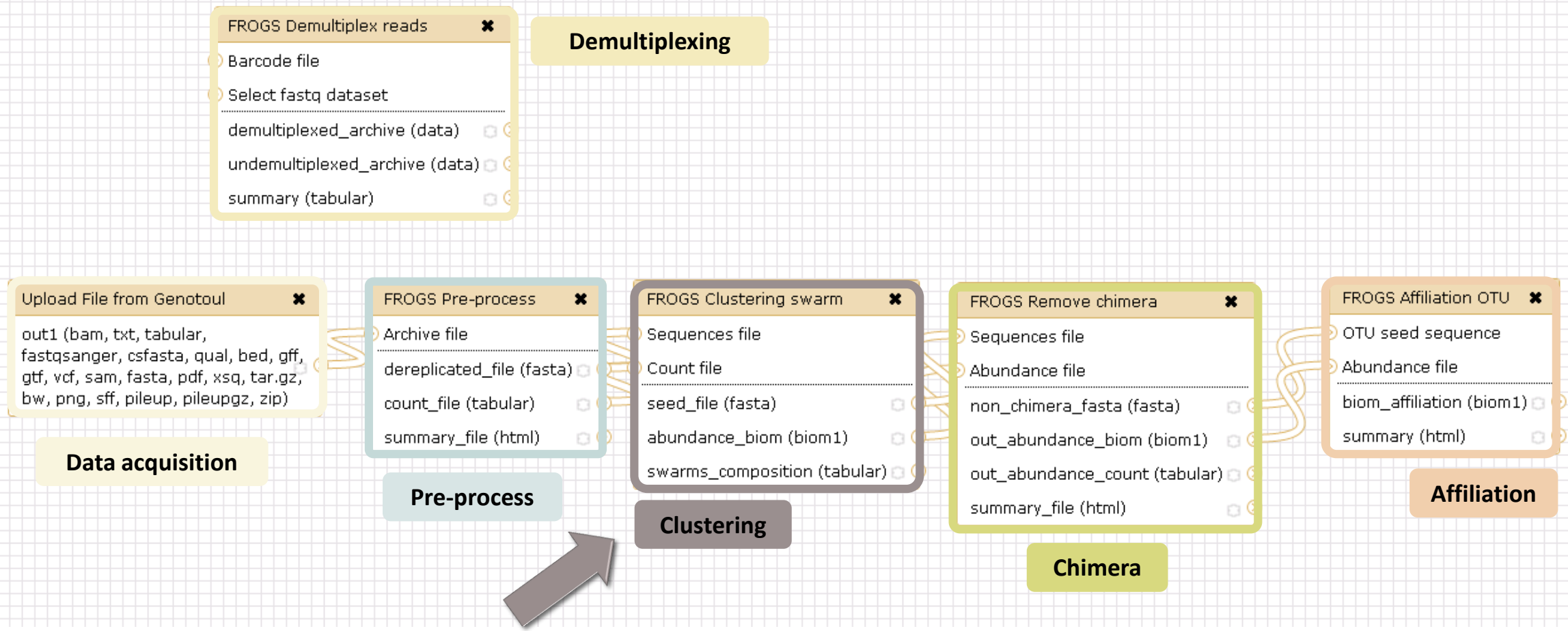
Supported since version 3.0

Length of the sequenced target < the sum of the lengths of the two reads

Length of the sequenced target \geq the sum of the lengths of the two reads

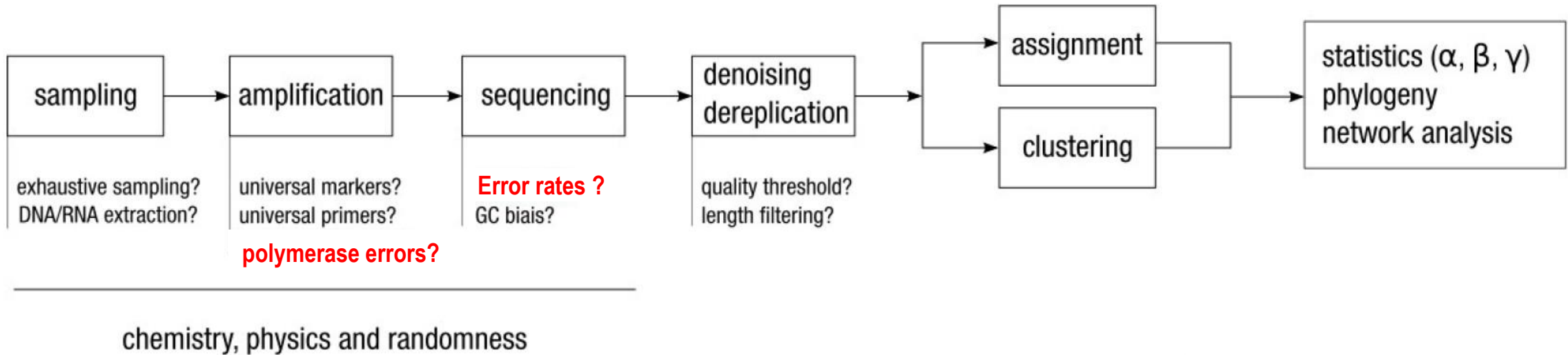
Supported since version 3.0 with option "keep unmerged reads" in preprocess Tool

Clustering tool



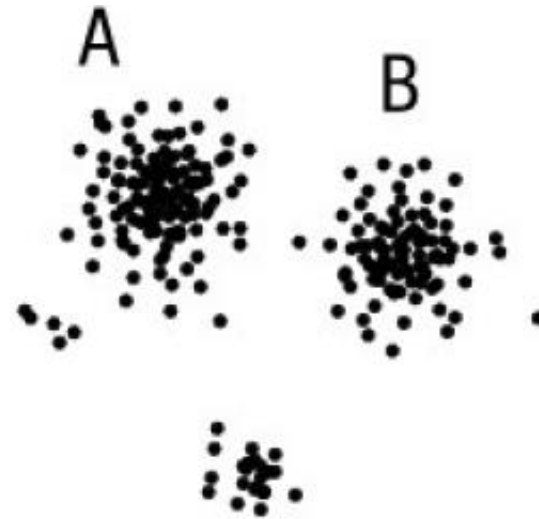
Why do we need clustering ?

Amplification and sequencing and are not perfect processes





Expected

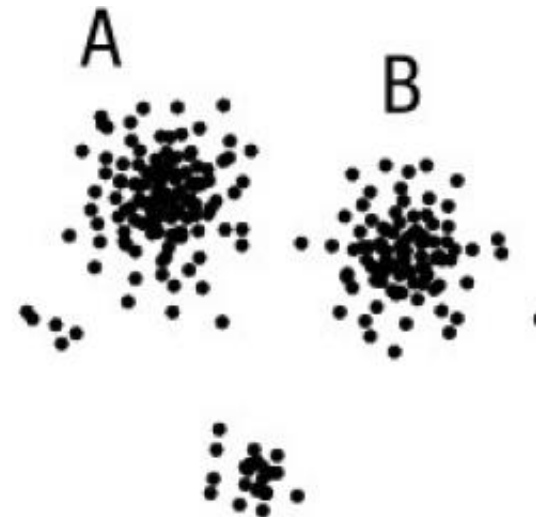


Results

Natural variability?
Technical noise?
Contaminant?
Chimeras?



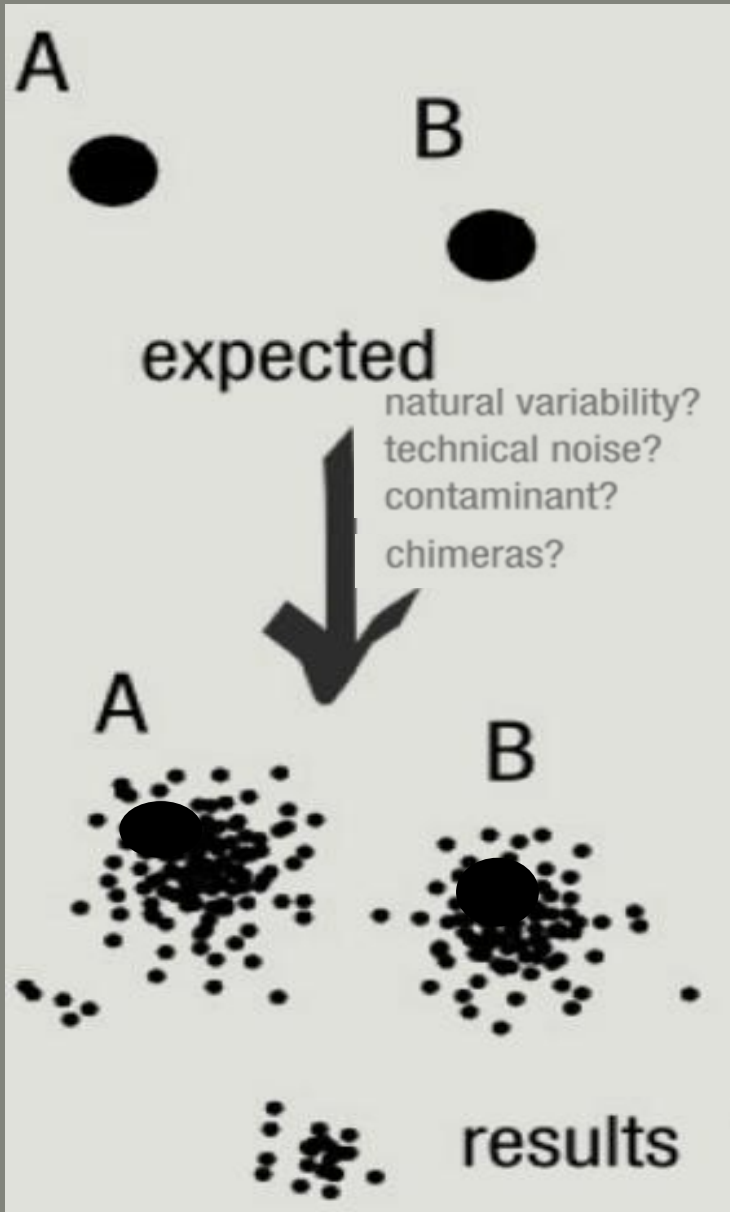
Expected



Results

Natural variability ?
Technical noise?
Contaminant?
Chimeras?

16S variability
Cf. RRNDB (ribosomal RNA operons database)
max. 21 copies of 16S in bacteria (*Photobacterium damsela*)
ex. *E. coli* 7 copies



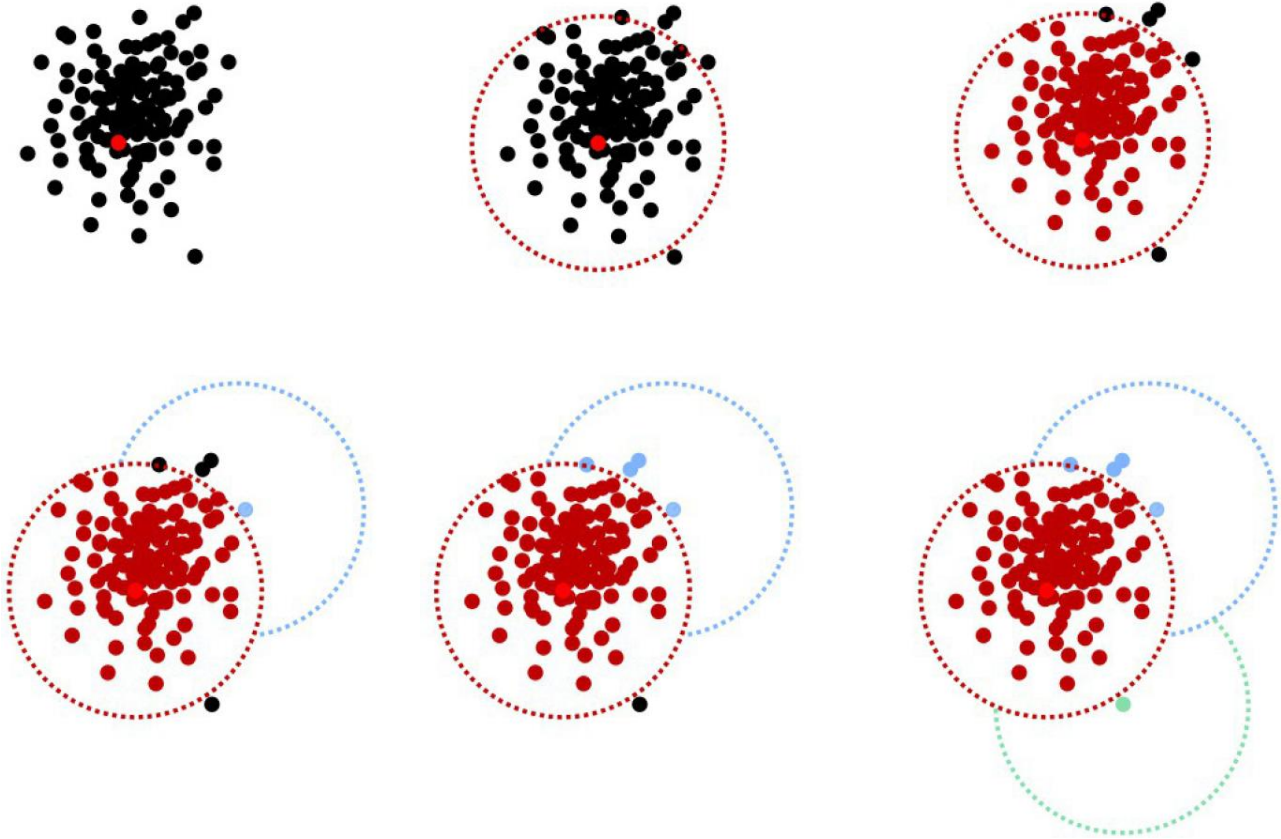
To have the best accuracy:

Method: All against all

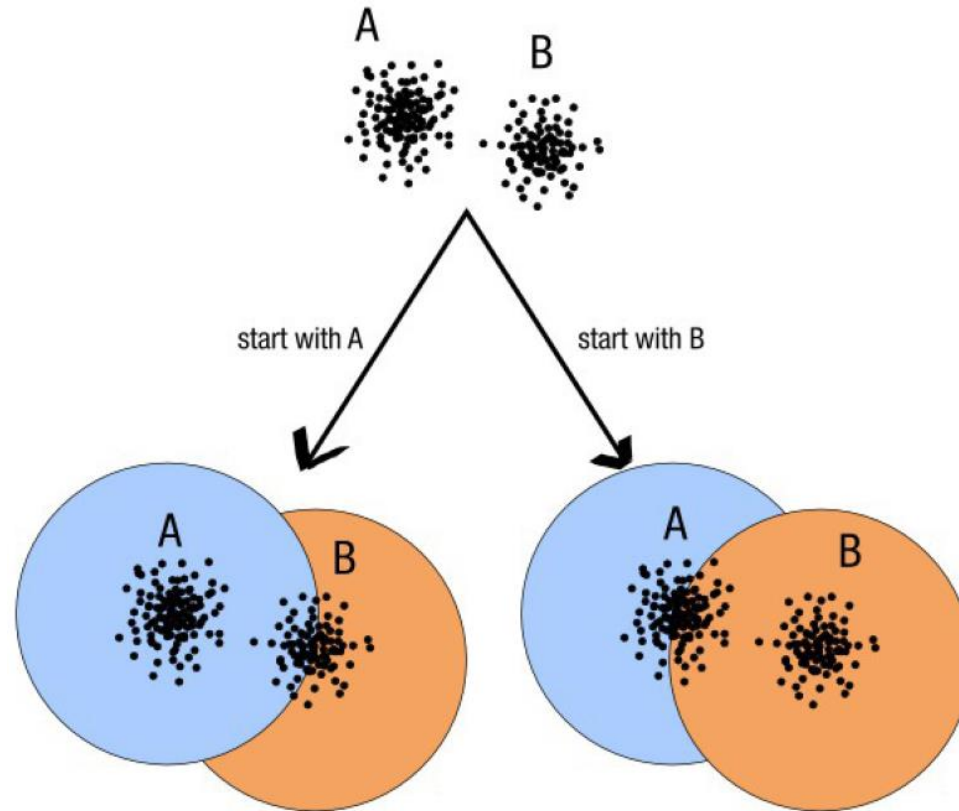
- Very accurate
- Requires a lot of memory and/or time

=> Impossible on very large datasets without strong filtering or sampling

How traditional clustering works ?

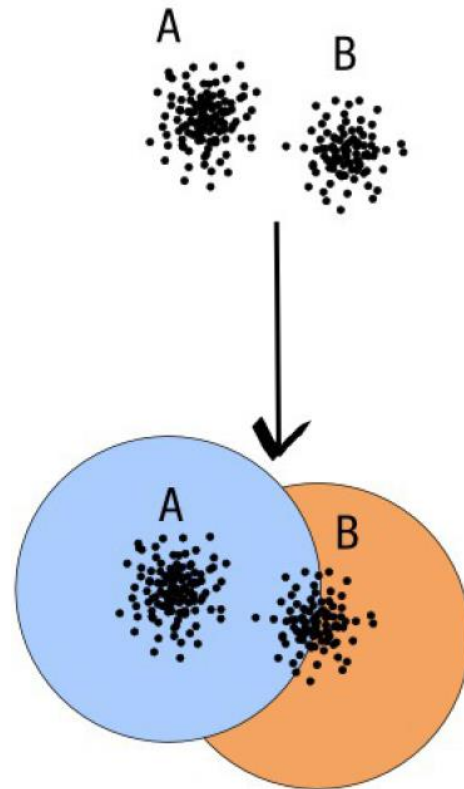


Input order dependent results

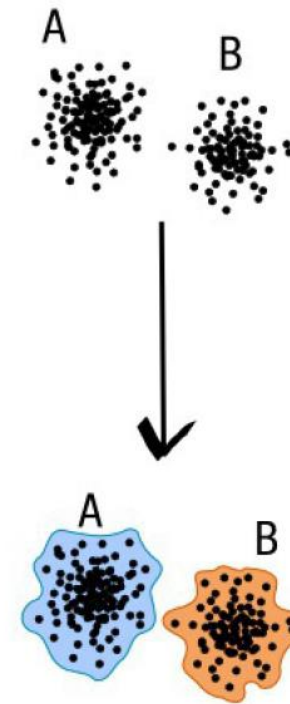


decreasing length,
decreasing abundance,
external references

Single a priori clustering threshold



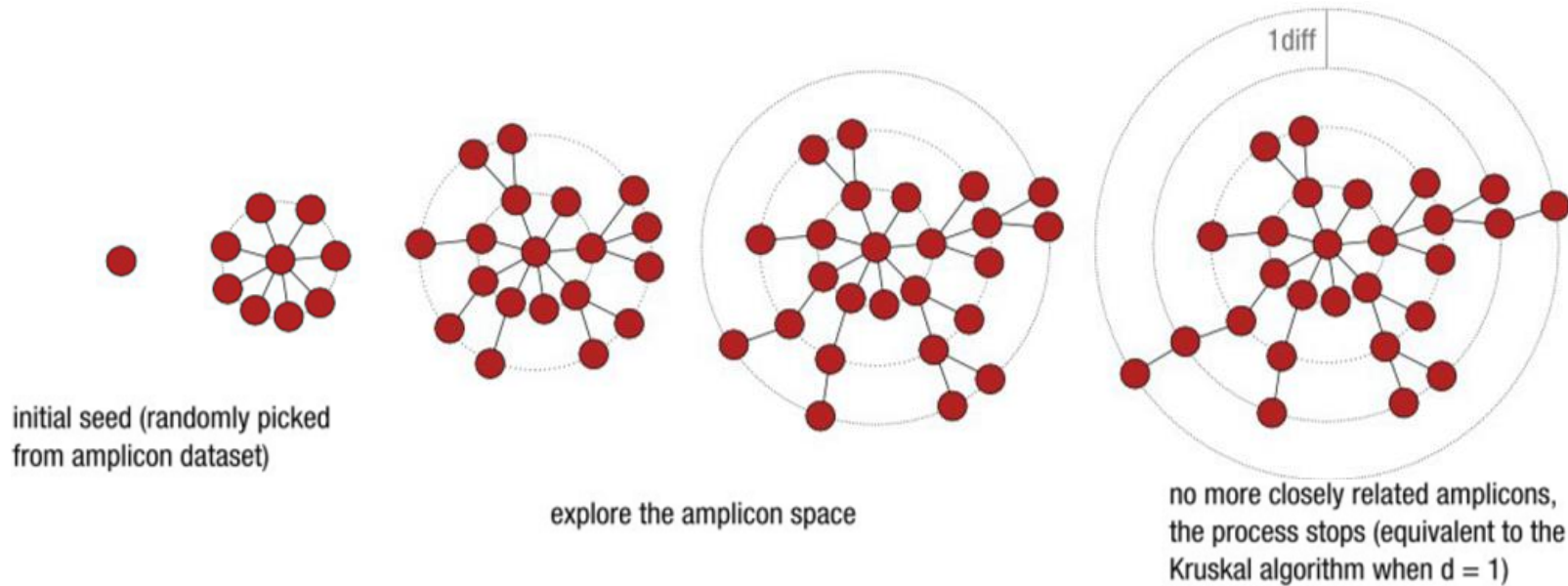
compromise threshold
unadapted threshold



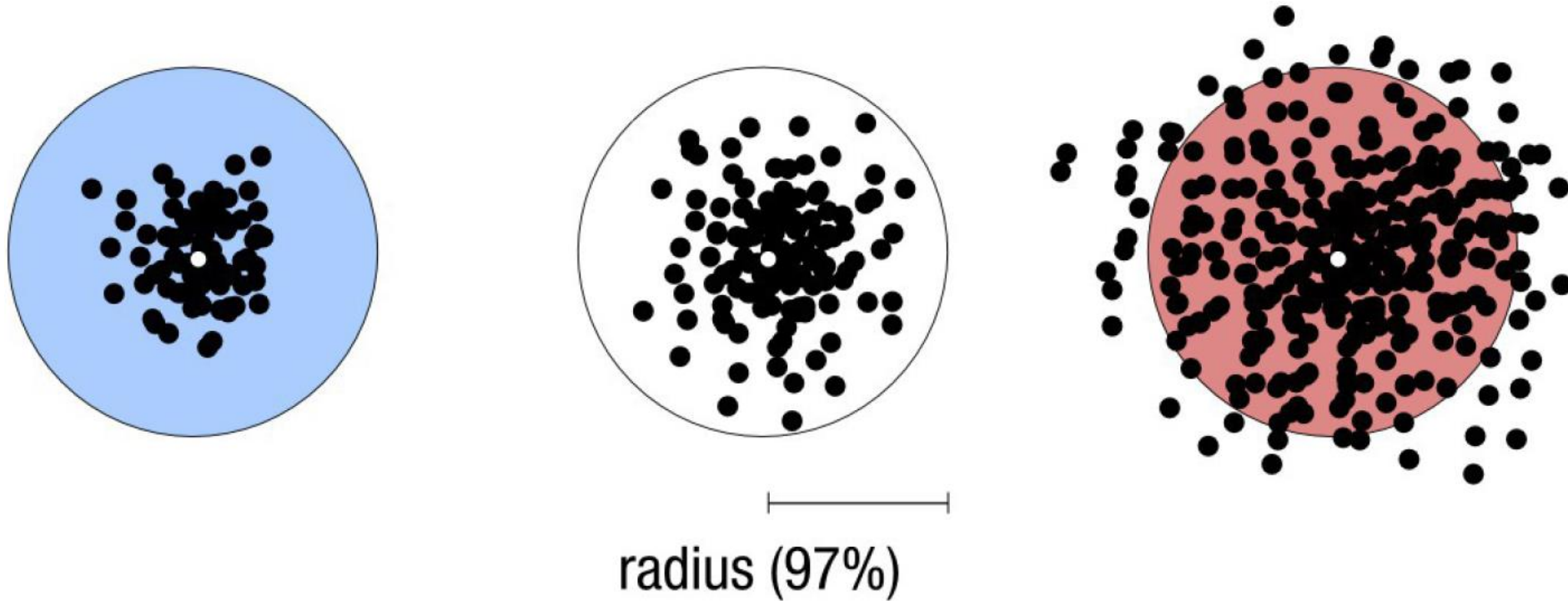
natural limits of clusters

Swarm clustering method

	ACGT	ACGT	ACGT
	AGGT	A - GT	A - - T
differences	1	1	2

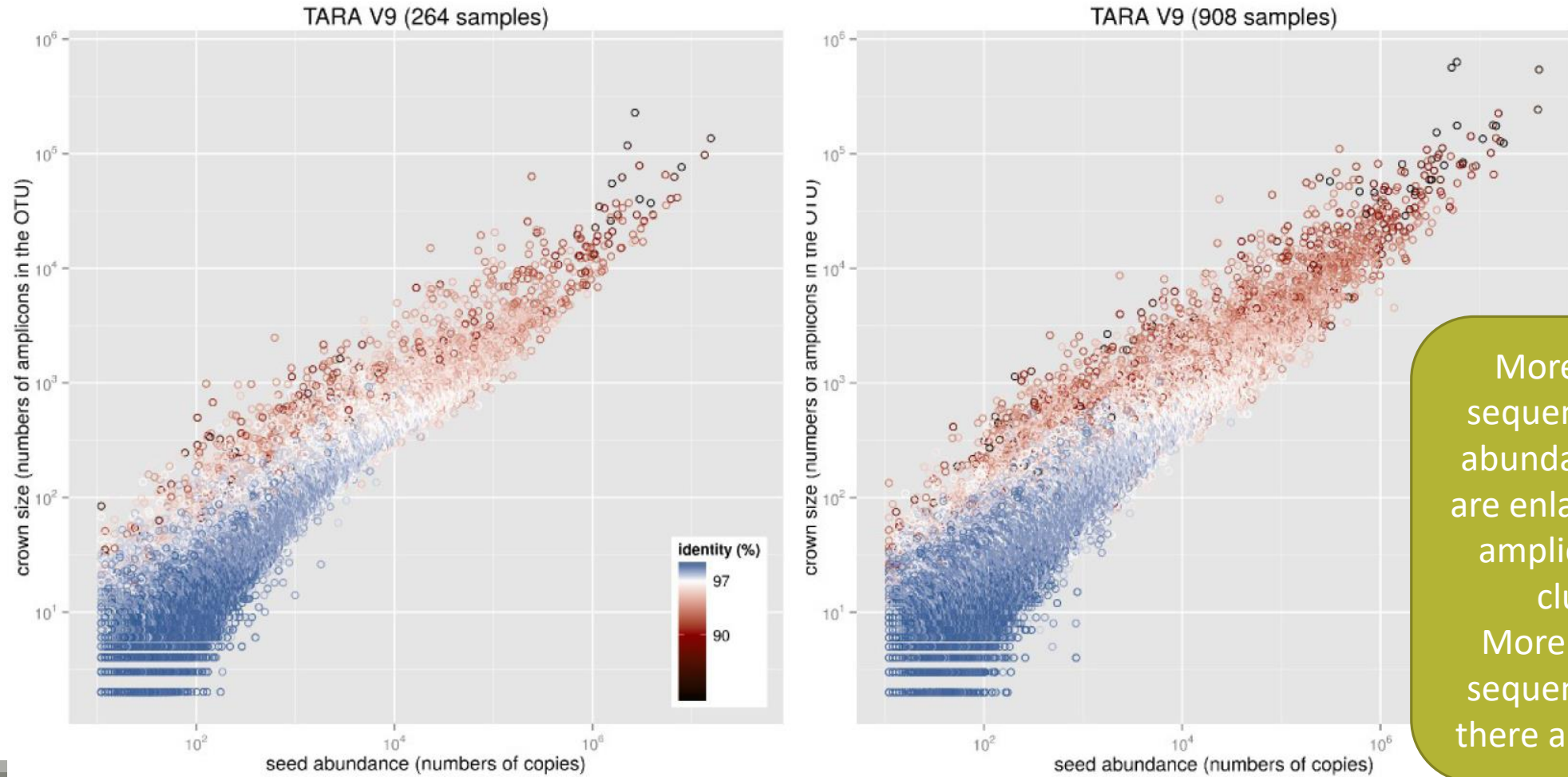


Comparison Swarm and 3% clusterings



Radius expressed as a percentage of identity with the central amplicon (97% is by far the most widely used clustering threshold)

Comparison Swarm and 3% clusterings



SWARM

A **robust** and **fast** clustering method for amplicon-based studies.

The purpose of **swarm** is to provide a novel clustering algorithm to handle **large sets of amplicons**.

swarm results are **resilient to input-order changes** and rely on a **small local linking threshold d** , the maximum number of differences between two amplicons.

swarm forms stable high-resolution clusters, with a high yield of biological information.

Swarm: robust and fast clustering method for amplicon-based studies.
Mahé F, Rognes T, Quince C, de Vargas C, Dunthorn M.
PeerJ. 2014 Sep 25;2:e593. doi: 10.7717/peerj.593. eCollection 2014.
PMID:25276506

FROGS Clustering swarm ✕

- Sequences file
- Count file
- abundance_biom (txt) ⊞
- seed_file (fasta) ⊞
- swarms_composition (tabular) ⊞

Clustering

FROGS Clustering swarm amplicon sequence clustering. (Galaxy Version r3.0-1.4) Options

Sequences file
 53: FROGS Pre-process: dereplicated.fasta
 The sequences file (format: fasta).

Count file
 54: FROGS Pre-process: count.tsv
 It contains the count by sample for each sequence (format: TSV).

Efficient denoising ? (equals to a first clustering step with d=1)
 Yes No
 If checked, clustering will be perform in two steps, first with distance = 1 and then with aggregation distance of next input parameter

Aggregation distance clustering

 Maximum number of differences between sequences in each aggregation swarm step.

Execute

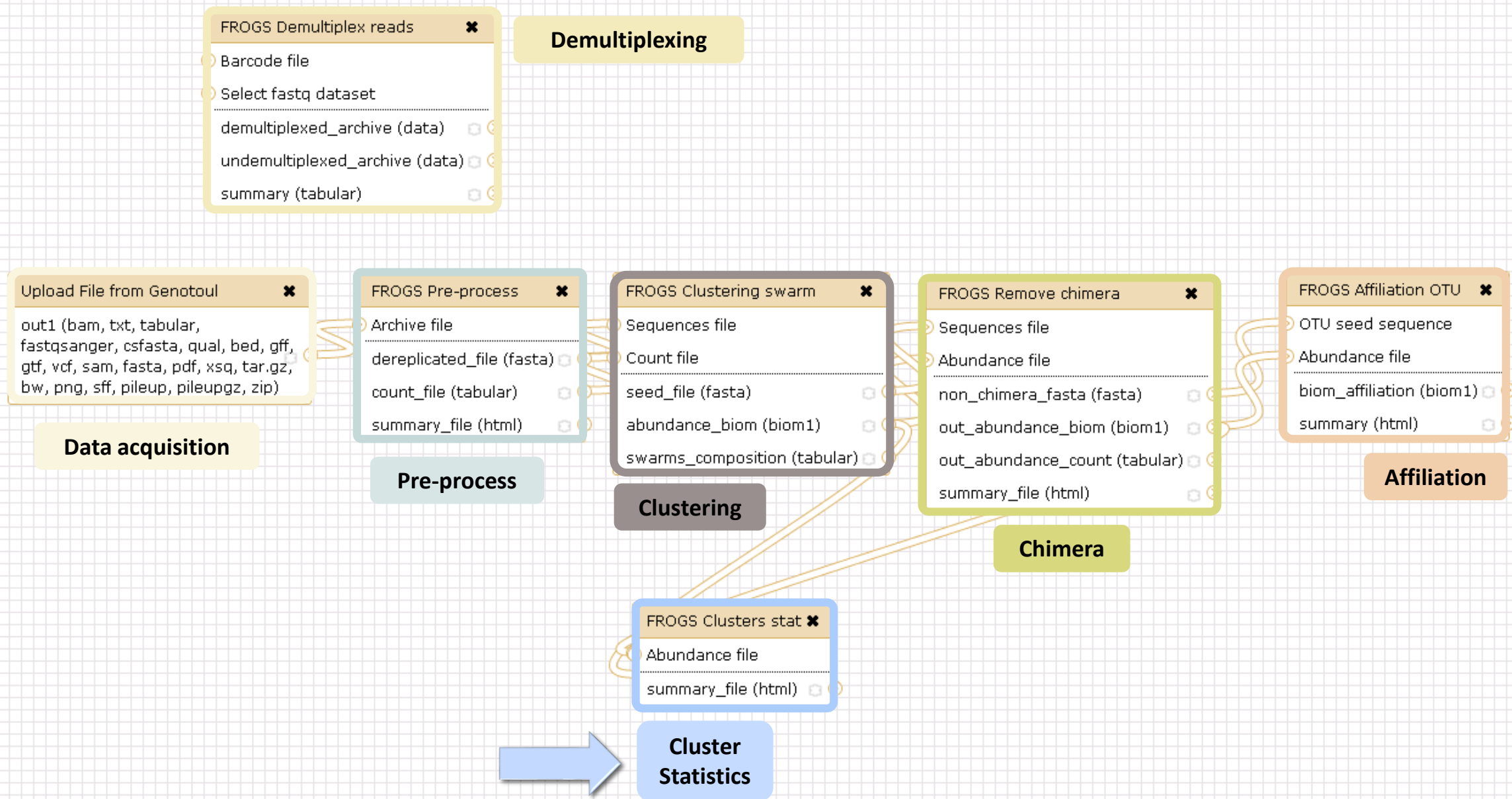


1st run for denoising:
 Swarm with $d = 1$ -> high clusters definition
 linear complexity

2nd run for clustering:
 Swarm with $d = 3$ on the **seeds** of first Swarm
 quadratic complexity




Gain time !
 Remove false positives !

Cluster stat tool



FROGS Clusters stat Process some metrics on clusters. (Galaxy Version 1.4.0) Options

Abundance file

   6: FROGS Clustering swarm: abundance.biom

Clusters abundance (format: BIOM).

Your Turn! - 3

LAUNCH CLUSTERING AND CLUSTERSTAT TOOLS

Exercise 3

Go to « [MiSeq merged](#) » history

Launch the Clustering SWARM tool on that data set with aggregation distance = 3 and the denoising

→ objectives :

- understand the denoising efficiency
- understand the ClusterStat utility

Exercise 3

1. How much time does it take to finish?
2. How many clusters do you get ?

Exercise 3

3. Launch FROGS Cluster Stat tools on the previous abundance biom file

FROGS Clusters stat Process
some metrics on clusters.

Exercise 3

4. Interpret the boxplot: **Clusters size summary**
5. Interpret the table: **Clusters size details**
6. What can we say by observing the **sequence distribution**?
7. How many clusters share “sampleB3” with at least one other sample?
8. How many clusters could we expect to be shared ?
9. How many sequences represent the 550 specific clusters of “sampleC2”?
10. This represents what proportion of “sampleC2”?
11. What do you think about it?
12. How do you interpret the « Hierarchical clustering » ?

The « Hierarchical clustering » is established with a Bray Curtis distance particularly well adapted to abundance table of very heterogenous values (very big and very small figures).

Tools

matrix

FROGS - Find Rapidly Otu with Galaxy Solution**OTUS RECONSTRUCTION**

FROGS Demultiplex reads
Attribute reads to samples in function of inner barcode.

FROGS Pre-process merging, denoising and dereplication.

FROGS Clustering swarm
amplicon sequence clustering.

FROGS Remove chimera
Remove PCR chimera in each sample.

FROGS Filters Filters OTUs on several criteria.

FROGS ITSx Extract the highly variable ITS1 and ITS2 subregions from ITS sequences.

FROGS Affiliation OTU
Taxonomic affiliation of each OTU's seed by RDPtools and BLAST

FROGS Clusters stat Process some metrics on clusters.

FROGS Affiliations stat
Process some metrics on taxonomies.

FROGS Affiliation postprocess
Optionnal step to resolve inclusive amplicon ambiguities and to aggregate OTUs based on alignment metrics

FROGS BIOM to std BIOM
Converts a FROGS BIOM in fully compatible BIOM.

FROGS BIOM to TSV Converts a BIOM file in TSV file.

FROGS TSV to BIOM
Converts a TSV file in a BIOM file.

Clusters distribution

Sequences distribution

Samples distribution

Clusters

5,940

Sequences

89,739

Most of clusters are singletons

Clusters size summary



Decile	Value
Min	1
1	1
2	1
3	1
4	1
Median	1
6	1
7	1
8	2
9	2
Max	13,337

History

chimera: report.html

13: FROGS Remove chimera:
non_chimera_abundance.biom

12: FROGS Remove chimera:
non_chimera.fasta

11: FROGS Clusters stat: summary.html
186.7 KB

format: html, database: ?

```
## Application
Software :/galaxydata/galaxy-prod/my_tools/FROGS
/app/clusters_stat.py (version : r3.0-3.0)
Command : /galaxydata/galaxy-prod/my_tools/FROGS
/app/clusters_stat.py --input-biom /galaxydata/galaxy-prod/my_files/000/330
/dataset_330065.dat --out
```

HTML file

7: FROGS Clustering swarm:
swarms_composition.tsv

6: FROGS Clustering swarm:
abundance.biom

5: FROGS Clustering swarm:
seed_sequences.fasta

4: FROGS Pre-process:
report.html

3: FROGS Pre-process:
count.tsv

2: FROGS Pre-process:
dereplicated.fasta

1: /work/project/frogs/Formation

Clusters size details

Most of clusters are singletons

CSV

Show 10 entries

Search:

Cluster size	Number of cluster	% of all clusters
1	4,595	77.36
2	865	14.56
3	154	2.59
4	84	1.41
5	42	0.71
6	29	0.49
7	23	0.39
8	13	0.22
9	6	0.10
10	6	0.10

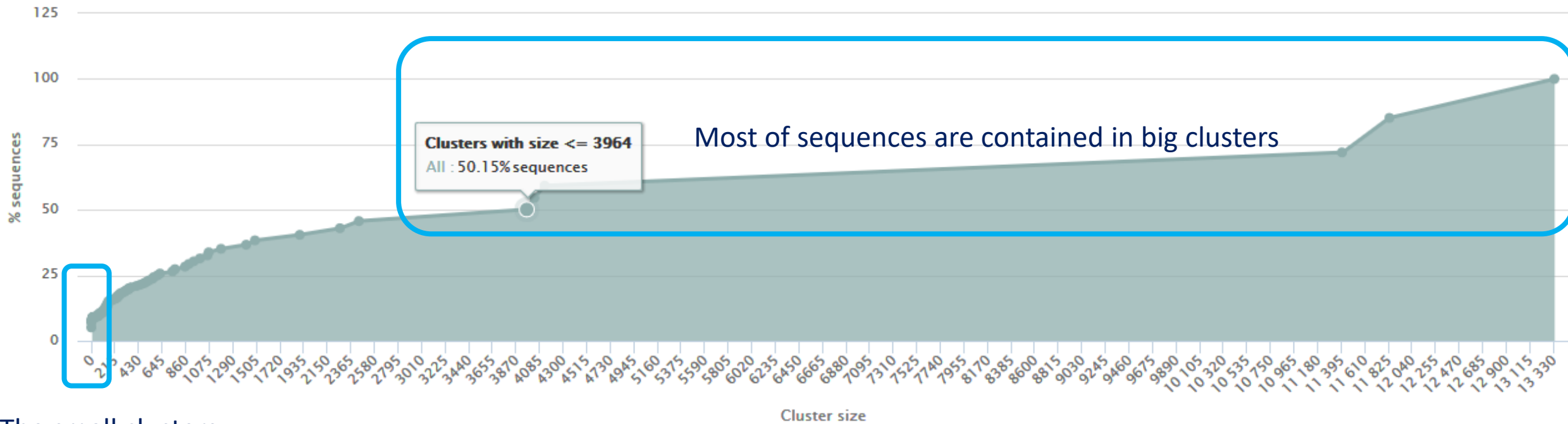
After clustering

Clusters distribution

Sequences distribution

Samples distribution

Cumulative sequences proportion by cluster size



Most of sequences are contained in big clusters

Clusters with size ≤ 3964
All : 50.15% sequences

The small clusters represent few sequences

N.B.: Select area to zoom in.

Sequences count 368 clusters of sampleA1 are common at least once with another sample

58 % of the specific clusters of sampleA1 represent around 5% of sequences
Could be interesting to remove if individual variability is not the concern of user

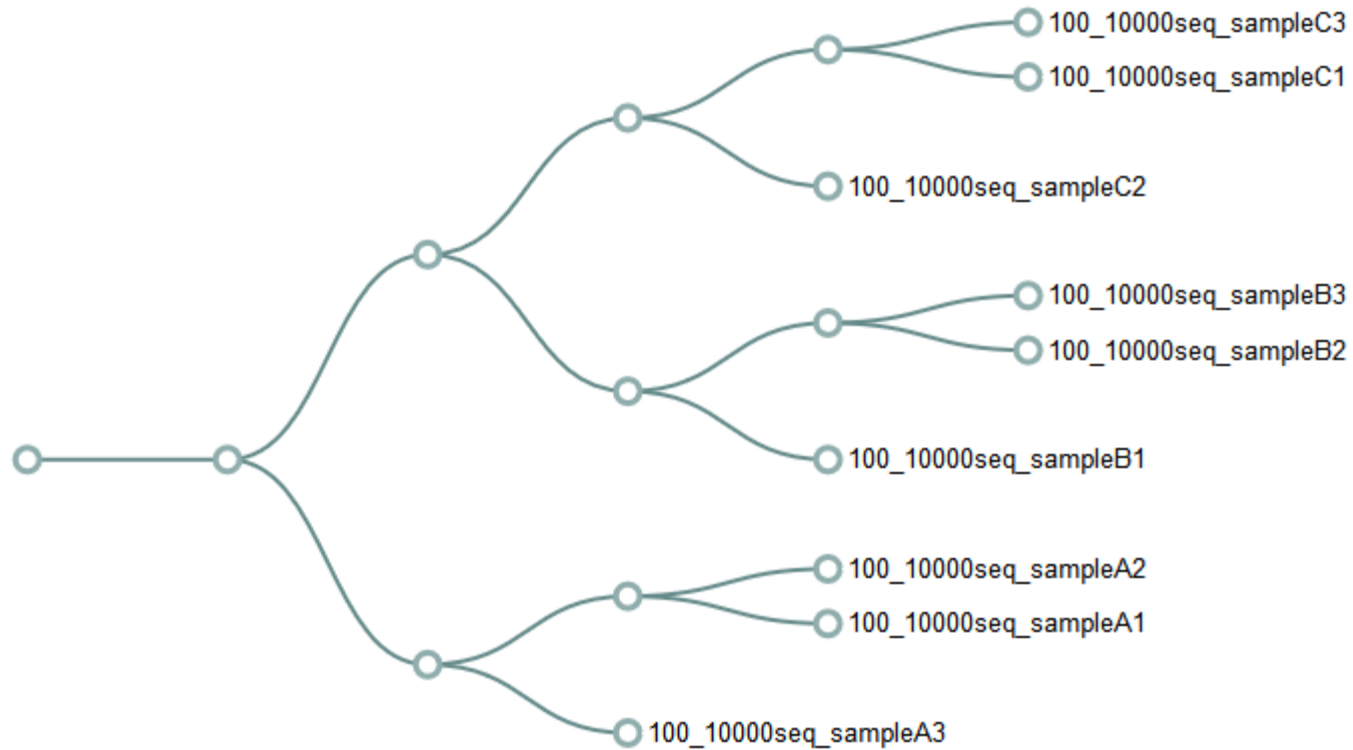
Show 10 entries

Sample	Total clusters	Shared clusters	Own clusters	Total sequences	Shared sequences	Own sequences
100_10000seq_sampleA1	881	368	513	9,975	9,447	528
100_10000seq_sampleA2	856	366	490	9,979	9,476	503
100_10000seq_sampleA3	867	384	483	9,972	9,478	494
100_10000seq_sampleB1	942	394	548	9,969	9,397	572
100_10000seq_sampleB2	881	373	508	9,970	9,455	515
100_10000seq_sampleB3	941	379	562	9,967	9,388	579
100_10000seq_sampleC1	910	371	539	9,965	9,413	552
100_10000seq_sampleC2	938	388	550	9,975	9,408	567
100_10000seq_sampleC3	878	362	516	9,967	9,442	525

Showing 1 to 9 of 9 entries

Previous 1 Next

Hierarchical clustering



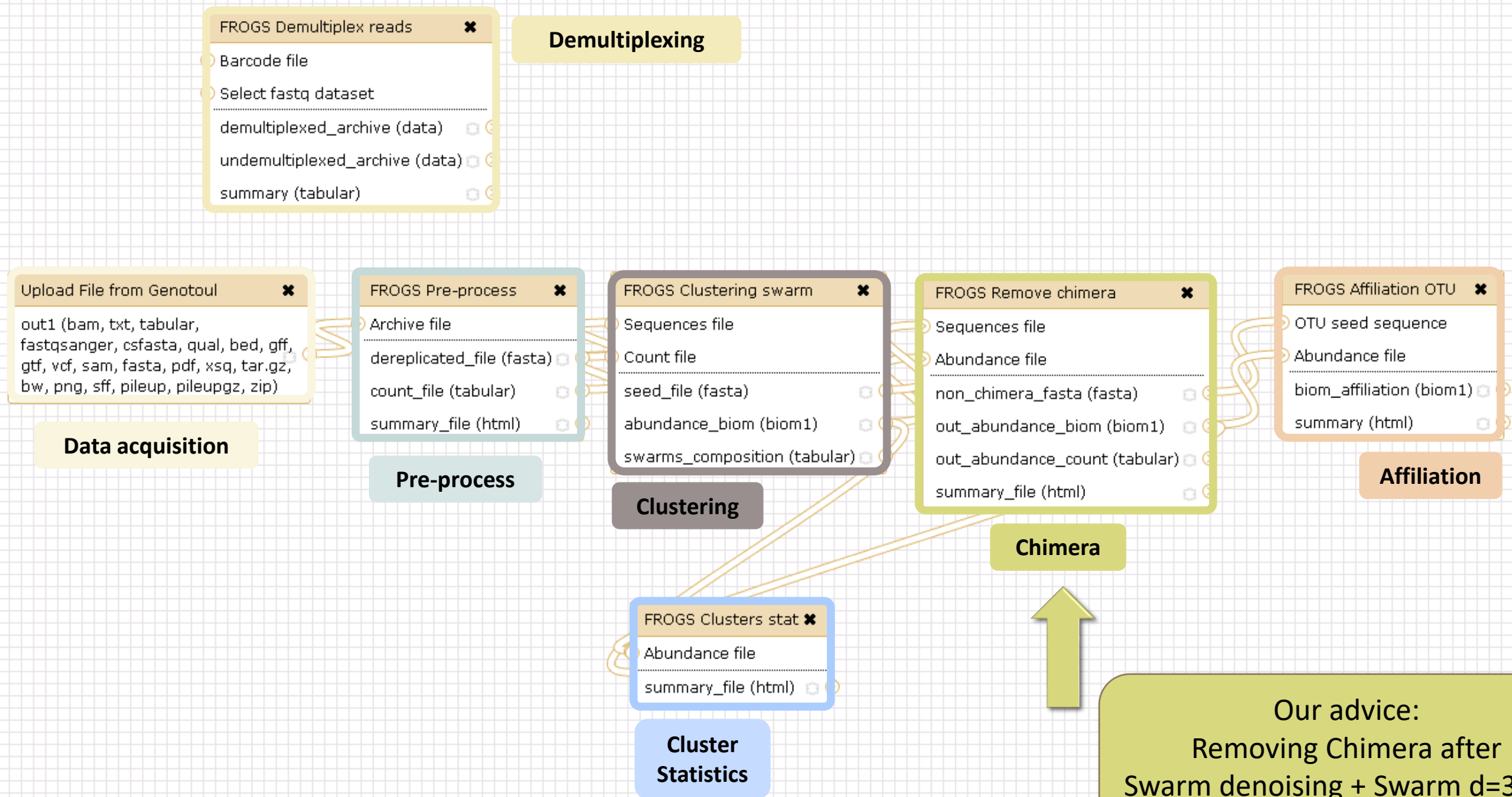
Hierarchical classification
on Bray Curtis distance

Newick tree available too

Newick

```
(((100_10000seq_sampleA3,(100_10000seq_sampleA1,100_10000seq_sampleA2):0.096):0.100,((100_10000seq_sampleB1,  
(100_10000seq_sampleB2,100_10000seq_sampleB3):0.101):0.102,(100_10000seq_sampleC2,(100_10000seq_sampleC1,100_10000seq_sampleC3):0.098):0.105):0.830):0.883);
```

Chimera removal tool



Our advice:
 Removing Chimera after
 Swarm denoising + Swarm d=3,
 for saving time without sensitivity loss

What is chimera ?

PCR-generated chimeras are typically created when an aborted amplicon acts as a primer for a heterologous template. Subsequent chimeras are about the same length as the non-chimeric amplicon and contain the forward (for.) and reverse (rev.) primer sequence at each end of the amplicon.

Chimera: from 5 to 45% of reads (Schloss 2011)

aborted amplification



next cycle's "primer"



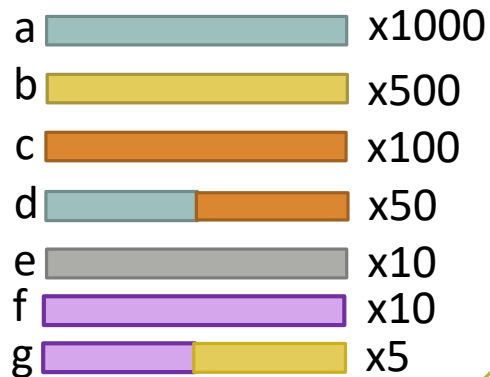
chimeric sequence



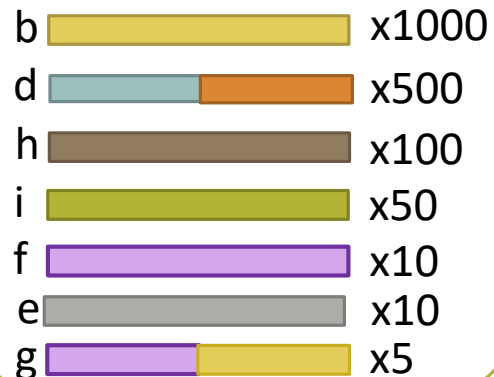
A smart removal chimera to be accurate

We use a sample cross-validation

Sample A



Sample B



“d” is view as chimera by Vsearch
Its “parents” are presents

“d” is view as normal sequence by Vsearch
Its “parents” are absents



- ⇒ For FROGS “d” is not a chimera
- ⇒ For FROGS “g” is a chimera, “g” is removed
- ⇒ FROGS increases the detection specificity

Your Turn! - 4

LAUNCH THE REMOVE CHIMERA TOOL

Exercise 5

Go to « [MiSeq merged](#) » history

Launch the « FROGS Remove Chimera » tool

Follow by the « FROGS ClusterStat » tool on the swarm d1d3 non chimera abundance biom

→ objectives :

- understand the efficiency of the chimera removal
- make links between small abundant OTUs and chimeras

FROGS Remove chimera ✕

- Sequences file
- Abundance file

- non_chimera_fasta (fasta)
- out_abundance_biom (biom1)
- out_abundance_count (tabular)
- summary_file (html)

Chimera

FROGS Remove chimera Step 3 in metagenomics analysis : Remove PCR chimera in each sample. (Galaxy Version 1.3.0) Options

Sequences file

5: FROGS Clustering swarm: seed_sequences.fasta

The sequences file (format: fasta).

Abundance type

BIOM file

Select the type of file where the abundance of each sequence by sample is stored.

Abundance file

6: FROGS Clustering swarm: abundance.biom

It contains the count by sample for each sequence.

Execute

Exercise 4

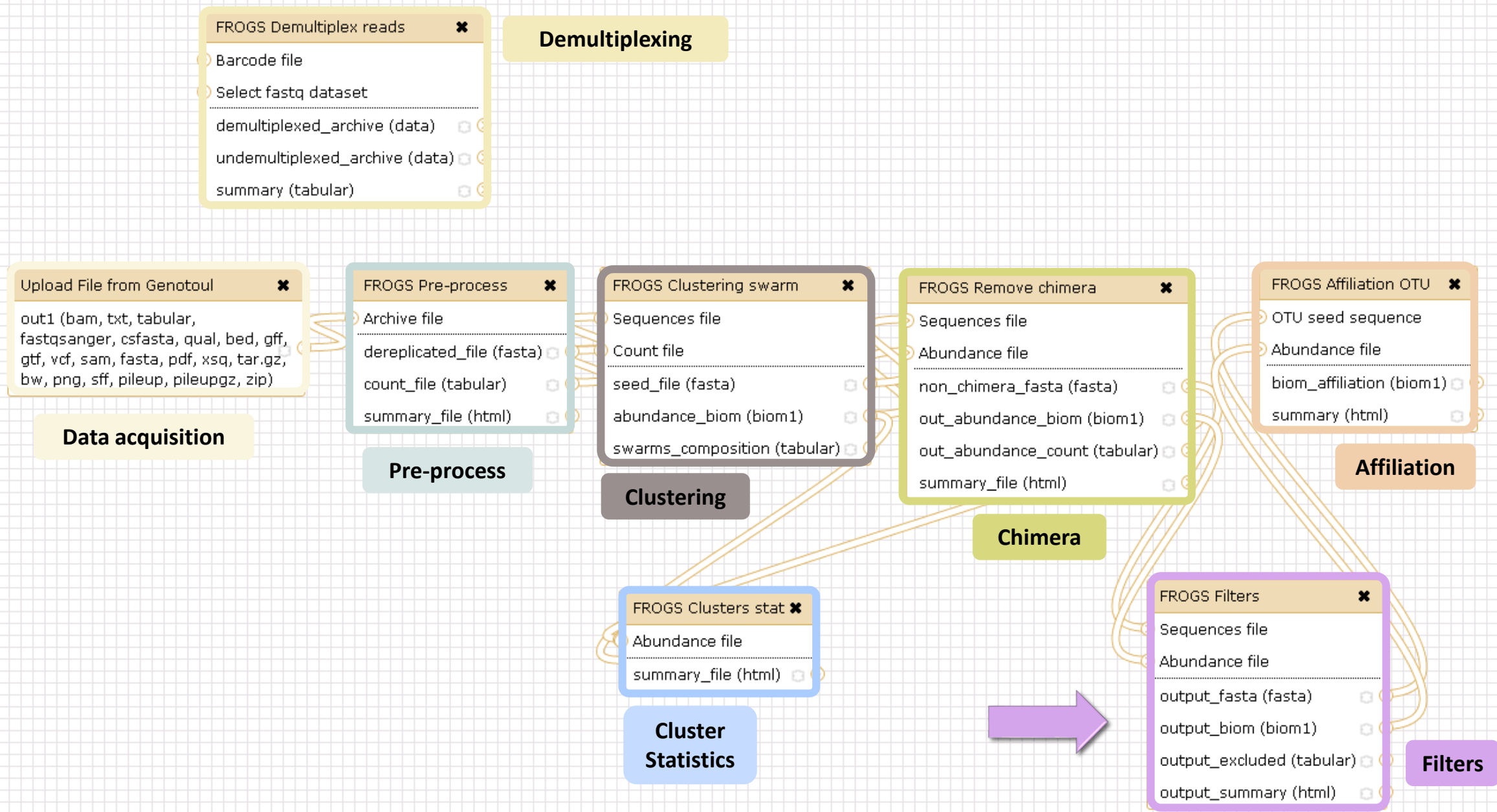
1. Understand the « FROGS remove chimera : report.html»
 - a. How many clusters are kept after chimera removal?
 - b. How many sequences that represent ? So what abundance?
 - c. What do you conclude ?

Exercise 4

2. Launch « FROGS ClusterStat » tool on non_chimera_abundance.biom
3. Rename output in summary_nonchimera.html
4. Compare the HTML files
 - a. Of what are mainly composed singleton ? (compare with previous summary.html)
 - b. What are their abundance?
 - c. What do you conclude ?

The weakly abundant Clusters are mainly false positives, our data would be much more exact if we remove them

Filters tool



Affiliation runs long time

Advise:

Apply filters between “Chimera Removal ” and “Affiliation”.
Remove OTUs with weak abundance and non redundant before affiliation.

You will gain time !

Filters

Filters allows to filter the result thanks to different criteria et may be used after different steps of pipeline :

- On the abundance
- On RDP affiliation
- On Blast affiliation
- On phix contaminant

After Affiliation tool

FROGS Filters ✕

- Sequences file
- Abundance file
- output_fasta (fasta) 🗑️
- output_biom (biom1) 🗑️
- output_excluded (tabular) 🗑️
- output_summary (html) 🗑️

Filters

4 filter sections

FROGS Filters Filters: OTUs on several criteria. (Galaxy Version 1.2.0) Options

Sequences file

 The sequence file to filter (format: fasta).

Abundance file

 The abundance file to filter (format: BIOM).

***** THE FILTERS ON OTUS IN SAMPLES, OTUS SIZE and SEQUENCE PERCENTAGE**

Apply filters
 If you want to filter OTUs on their abundance and occurrence.

Minimum number of samples

 Fill the field only if you want this treatment. Keep OTU present in at least this number of samples.

Minimum proportion/number of sequences to keep OTU

 Fill the field only if you want this treatment. Use decimal notation for proportion (example: 0.01 for keep OTU with at least 1% of all sequences) ; Use integer notation for number of sequence (example: 2 for keep OTU with at least 2 sequences, so remove single singleton).

N biggest OTU

 Fill the fields only if you want this treatment. Keep the N biggest OTU.

***** THE FILTERS ON RDP**

Apply filters
 If you want to filter OTUs on their taxonomic affiliation produced by RDP.

Rank with the bootstrap filter

Minimum bootstrap % (between 0 and 1)

***** THE FILTERS ON BLAST**

Apply filters
 If you want to filter OTUs on their taxonomic affiliation produced by Blast.

Maximum e-value (between 0 and 1)

 Fill the field only if you want this treatment

Minimum identity % (between 0 and 1)

 Fill the field only if you want this treatment

Minimum coverage % (between 0 and 1)

 Fill the field only if you want this treatment

Minimum alignment length

 Fill the field only if you want this treatment

***** THE FILTERS ON CONTAMINATIONS**

Apply filters
 If you want to filter OTUs on classical contaminations.

Cotaminant databank

 The phiX databank (the phiX is a control added in Illumina sequencing technologies).

Abundance filters

RDP affiliation filters

BLAST affiliation filters

Contamination filter

Input

FROGS Filters Filters OTUs on several criteria. (Galaxy Version 1.2.0) Options

Sequences file

The sequence file to filter (format: fasta).

Abundance file

The abundance file to filter (format: BIOM).

Fasta sequences and its corresponding abundance biom files

Filter 1 : abundance

***** THE FILTERS ON OTUS IN SAMPLES, OTUS SIZE and SEQUENCE PERCENTAGE**

Apply filters

If you want to filter OTUs on their abundance and occurrence.

Minimum number of samples

Fill the field only if you want this treatment. Keep OTU present in at least this number of samples.

Minimum proportion/number of sequences to keep OTU

Fill the field only if you want this treatment. Use decimal notation for proportion (example: 0.01 for keep OTU with at least 1% of all sequences) ; Use integer notation for number of sequence (example: 2 for keep OTU with at least 2 sequences, so remove single singleton).

N biggest OTU

Fill the fields only if you want this treatment. Keep the N biggest OTU.

*** THE FILTERS ON RDP

Apply filters

If you want to filter OTUs on their taxonomic affiliation produced by RDP.

Rank with the bootstrap filter

Genus

Minimum bootstrap % (between 0 and 1)

0.8

Filter 2 & 3:
affiliation

*** THE FILTERS ON BLAST

Apply filters

If you want to filter OTUs on their taxonomic affiliation produced by Blast.

Maximum e-value (between 0 and 1)

Fill the field only if you want this treatment

Minimum identity % (between 0 and 1)

1

Fill the field only if you want this treatment

Minimum coverage % (between 0 and 1)

0.95

Fill the field only if you want this treatment

Minimum alignment length

Fill the field only if you want this treatment

Filter 4 :
contamination

Cotaminant databank

phIX



The phIX databank (the phIX is a control added in Illumina sequencing technologies).

Soon, several contaminant banks

Your Turn! - 5

LAUNCH THE « FILTERS » TOOL

Exercise 5

Go to history « **MiSeq merged** »

Launch « Filters » tool with non_chimera_abundance.biom, non_chimera.fasta

Apply 2 filters :

- **Minimum proportion/number of sequences to keep OTU: 0.00005***
- **Minimum number of samples: 3**

→ objective : play with filters, understand their impacts on false-positives OTUs

FROGS Filters ✕

- Sequences file
- Abundance file
- output_fasta (fasta) 🗑️
- output_biom (biom1) 🗑️
- output_excluded (tabular) 🗑️
- output_summary (html) 🗑️

Filters

FROGS Filters Filters OTUs on several criteria. (Galaxy Version 1.2.0) Options

Sequences file

 The sequence file to filter (format: fasta).

Abundance file

 The abundance file to filter (format: BIOM).

***** THE FILTERS ON OTUS IN SAMPLES, OTUS SIZE and SEQUENCE PERCENTAGE**

Apply filters
 If you want to filter OTUs on their abundance and occurrence.

Minimum number of samples

 Fill the field only if you want this treatment. Keep OTU present in at least this number of samples.

Minimum proportion/number of sequences to keep OTU

 Fill the field only if you want this treatment. Use decimal notation for proportion (example: 0.01 for keep OTU with at least 1% of all sequences) ; Use integer notation for number of sequence (example: 2 for keep OTU with at least 2 sequences, so remove single singleton).

N biggest OTU

 Fill the fields only if you want this treatment. Keep the N biggest OTU.

***** THE FILTERS ON RDP**

 If you want to filter OTUs on their taxonomic affiliation produced by RDP.

***** THE FILTERS ON BLAST**

 If you want to filter OTUs on their taxonomic affiliation produced by Blast.

***** THE FILTERS ON CONTAMINATIONS**

 If you want to filter OTUs on classical contaminations.

Output

- 92: FROGS Filters: report.html** 👁️ ✕
- 91: FROGS Filters: excluded.tsv** 👁️ ✕
- 90: FROGS Filters: abundance.biom** 👁️ ✕
- 89: FROGS Filters: sequences.fasta** 👁️ ✕



If Filters fields are « Apply » so you have to fill at one field. Otherwise, galaxy become red !

Exercise 5

1. What are the output files of “Filters” ?
2. Explore “FROGS Filter : report.html” file.
3. How many OTUs have you removed ?
4. Build the Venn diagram on the two filters.
5. How many OTUs have you removed with each filter “abundance > 0.005%”, “Remove OTUs that are not present at least in 3 samples”?
6. How many OTUs do they remain ?
7. Is there a sample more impacted than the others ?
8. To characterize these new OTUs, do not forget to launch “FROGS Cluster Stat” tool, and rename the output HTML file.

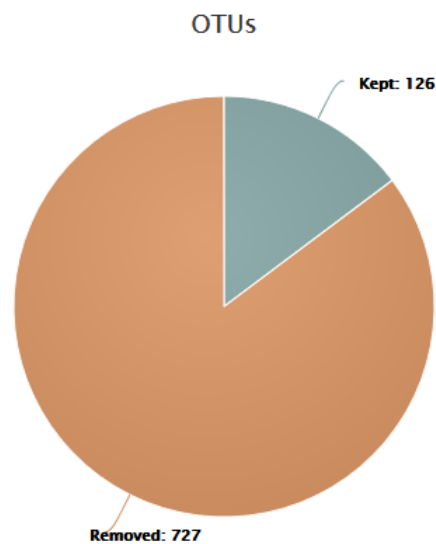
Filters by OTUs

Filters by samples

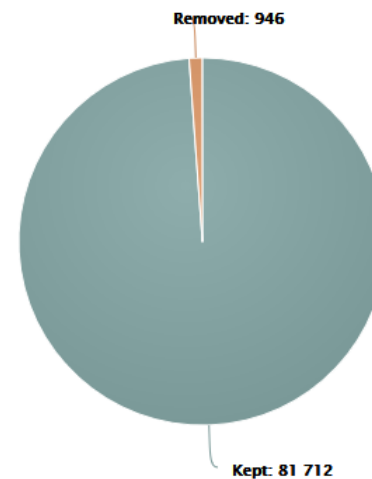


Configuration tabs

Filters summary



Abundance



Filters intersections

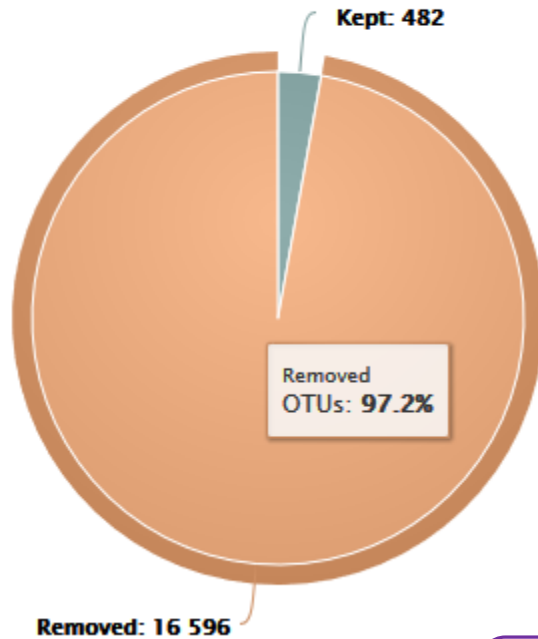
Draw a Venn to see which OTUs had been deleted by the filters chosen (Maximum 6 options):

- Present in minus of 3 samples
- Abundance < 5e-05

Venn

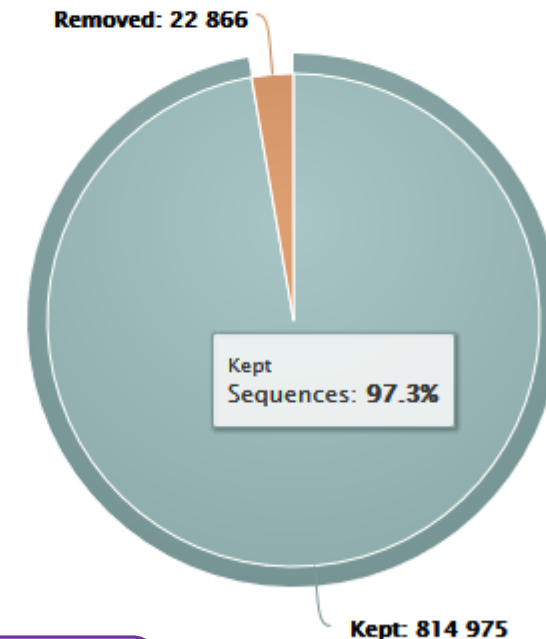
What are the remaining singletons ?

OTUs



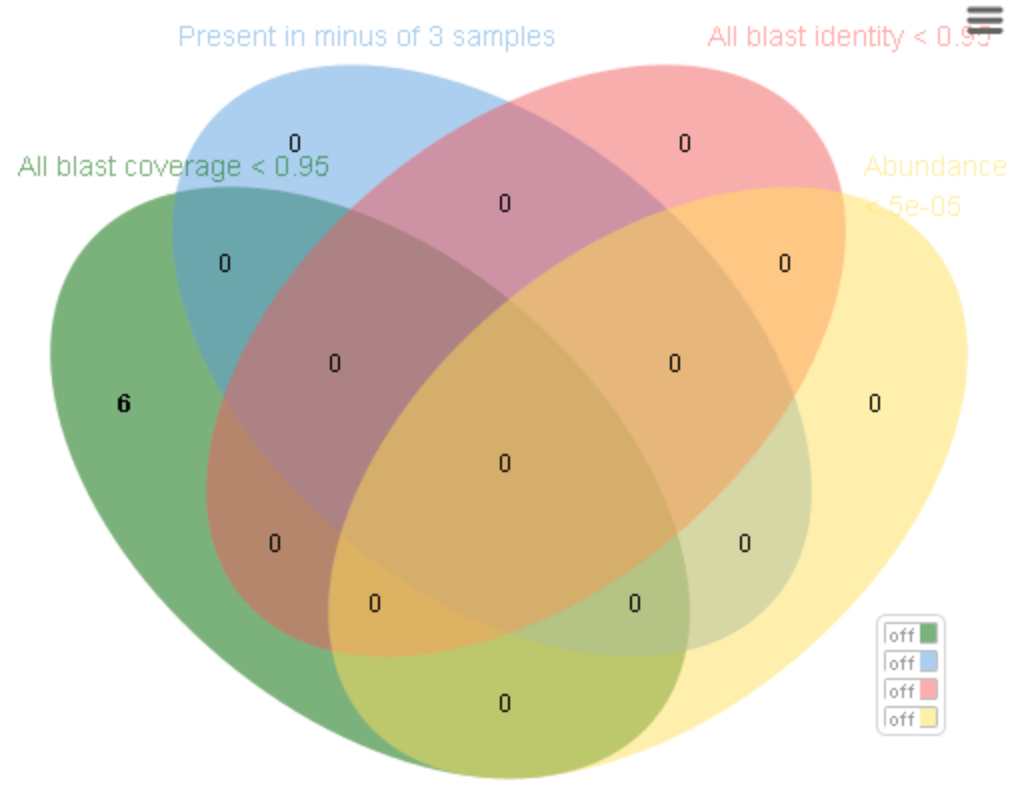
On simulated data, singleton are:
~99,9% are chimera
and
~0,1% are sequences with
sequencing errors, non clustered

Abundance



Removing little OTUs (conservation rate =0.005%)
and non shared OTU (in less than 2 samples)

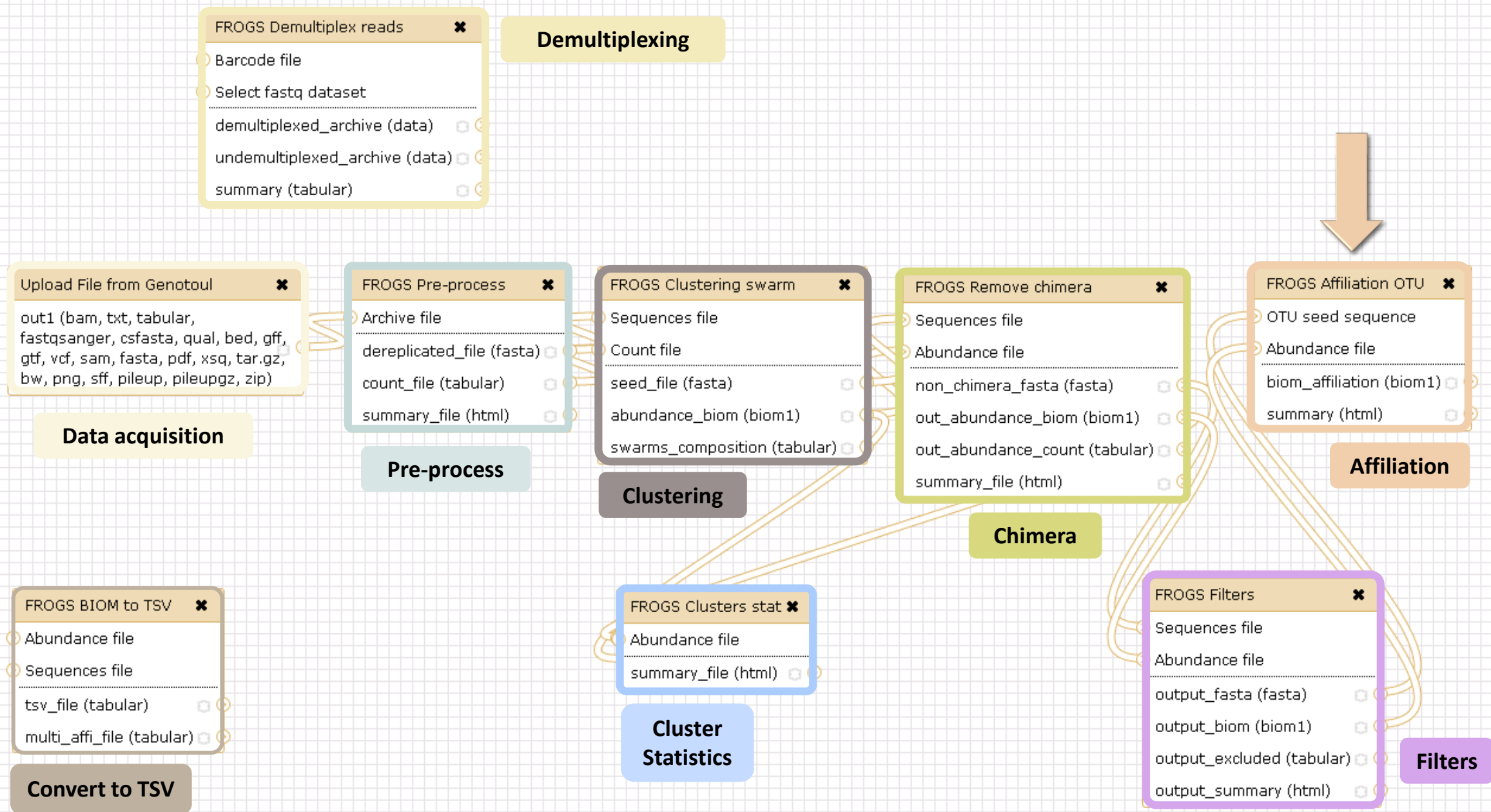
Venn on removed OTUs



- off
- off
- off
- off

Close

Affiliation tool



FROGS Affiliation OTU ✕

- OTU seed sequence
- Abundance file

- biom_affiliation (biom1) 🗑
- summary (html) 🗑

Affiliation

FROGS Affiliation OTU Step 4 in metagenomics analysis : Taxonomic affiliation of each OTU's seed by RDPtools and BLAST Options
(Galaxy Version 0.8.0)

Using reference database
silva132 16S OR silva132 16S
Select reference from the list

Also perform RDP assignation?
 Yes No Optional
Taxonomy affiliation will be perform thanks to Blast. This o... form it also with RDP classifier (default No)

OTU seed sequence
📄 🗑 📁 17: FROGS Filters: sequences.fasta
OTU sequences (format: fasta).

Abundance file
📄 🗑 📁 18: FROGS Filters: abundance.biom
OTU abundances (format: BIOM).

Execute

- silva132 16S
- silva132_pintail100 16S
- silva132_pintail80 16S
- silva132_pintail50 16S
- silva132 18S
- silva132 23S
- silva128 16S
- silva128 23S
- silva123 16S
- silva123 23S
- silva123 18S
- greengenes13_5
- midas_S123_2.1.3
- midas_S119_1.20
- pr2_gb203_4.5
- rpoB_122017
- Unite_s_7.1_20112016

← For ITS

1 Cluster = 2 affiliations

1. RDPClassifier* (Ribosomal Database Project): one affiliation with bootstrap, on each taxonomic subdivision.

Bacteria(100);Firmicutes(100);Clostridia(100);Clostridiales(100);Lachnospiraceae(100);Pseudobutyrvibrio(80); Pseudobutyrvibrio xylanivorans (80)

2. NCBI Blastn+** : all identical Best Hits with identity %, coverage %, e-value, alignment length and a special tag “**Multi-affiliation**”.

Bacteria;Firmicutes;Clostridia;Clostridiales;Lachnospiraceae;Pseudobutyrvibrio;Pseudobutyrvibrio ruminis; Pseudobutyrvibrio xylanivorans

Identity: 100% and Coverage: 100%

* Appl. Environ. Microbiol. August 2007 vol. 73 no. 16 5261-5267. doi : 10.1128/AEM.00062-07
Naïve Bayesian Classifier for Rapid Assignment of rRNA Sequences into the New Bacterial Taxonomy.
Qiong Wang, George M.Garrity, James M. Tiedje and James R. Cole

** BMC Bioinformatics 2009, 10:421. doi:10.1186/1471-2105-10-421
BLAST+: architecture and applications

Christiam Camacho, George Coulouris, Vahram Avagyan, Ning Ma, Jason Papadopoulos, Kevin Bealer and Thomas L Madden

Affiliation Strategy of FROGS

Blastn+ with “**Multi-affiliation**” management

V3 – V4	Bacteria Firmicutes Clostridia Clostridiales Lachnospiraceae Pseudobutyrvibrio 16S unknown species
V3 – V4	Bacteria Firmicutes Clostridia Clostridiales Lachnospiraceae Pseudobutyrvibrio 16S Butyrvibrio fibrisolvens
V3 – V4	Bacteria Firmicutes Clostridia Clostridiales Lachnospiraceae Pseudobutyrvibrio 16S rumen bacterium 8 9293-9
V3 – V4	Bacteria Firmicutes Clostridia Clostridiales Lachnospiraceae Pseudobutyrvibrio 16S Pseudobutyrvibrio xylanivorans
V3 – V4	Bacteria Firmicutes Clostridia Clostridiales Lachnospiraceae Pseudobutyrvibrio 16S Pseudobutyrvibrio ruminis

5 identical blast best hits on SILVA 123 databank

Affiliation Strategy of FROGS

Blastn+ with “**Multi-affiliation**” management

V3 – V4	Bacteria Firmicutes Clostridia Clostridiales Lachnospiraceae Pseudobutyrvibrio 16S unknown species
V3 – V4	Bacteria Firmicutes Clostridia Clostridiales Lachnospiraceae Pseudobutyrvibrio 16S Butyrvibrio fibrisolvens
V3 – V4	Bacteria Firmicutes Clostridia Clostridiales Lachnospiraceae Pseudobutyrvibrio 16S rumen bacterium 8 9293-9
V3 – V4	Bacteria Firmicutes Clostridia Clostridiales Lachnospiraceae Pseudobutyrvibrio 16S Pseudobutyrvibrio xylanivorans
V3 – V4	Bacteria Firmicutes Clostridia Clostridiales Lachnospiraceae Pseudobutyrvibrio 16S Pseudobutyrvibrio ruminis



FROGS Affiliation: Bacteria | Firmicutes | Clostridia | Clostridiales | Lachnospiraceae | Pseudobutyrvibrio | **Multi-affiliation**

Your Turn! – 6

LAUNCH THE « FROGS AFFILIATION » TOOL

Exercise 6.1

Go to « **MiSeq merged** » history

Launch the « FROGS Affiliation » tool with

- **SILVA 123 or 128 or 132** 16S database
- FROGS Filters abundance biom and fasta files (after swarm d1+d3, remove chimera and filter low abundances)





→ objectives :

- understand abundance tables columns
- understand the BLAST affiliation

Miseq
merged

FROGS Affiliation OTU ✕

- OTU seed sequence
- Abundance file




- biom_affiliation (biom1)  
- summary (html)  




Affiliation

FROGS Affiliation OTU Step 4 in metagenomics analysis : Taxonomic affiliation of each OTU's seed by RDPtools and BLAST Options
(Galaxy Version 0.8.0)



Using reference database
silva123 16S ▼
Select reference from the list

Also perform RDP assignment?
 Yes No
Taxonomy affiliation will be perform thanks to Blast. This option allow you to perform it also with RDP classifier (default No)

OTU seed sequence
   17: FROGS Filters: sequences.fasta ▼
OTU sequences (format: fasta).

Abundance file
   18: FROGS Filters: abundance.biom ▼
OTU abundances (format: BIOM).

Exercise 6.1

1. What are the « FROGS Affiliation » output files ?
2. How many sequences are affiliated by BLAST ?
3. Click on the « eye » button on the BIOM output file, what do you understand ? 
4. Use the Biom_to_TSV tool on this last file and click again on the "eye" on the new output generated. 
 What do the columns ?
 What is the difference if we click on case or not ? What consequence about weight of your file ?

FROGS BIOM to TSV Converts a BIOM file in TSV file. (Galaxy Version 2.1.0) Options

Abundance file

 The BIOM file to convert (format: BIOM).

Sequences file

 The sequences file (format: fasta). If you use this option the sequences will be add in TSV.

Extract multi-alignments

 If you have used FROGS affiliation on your data, you can extract information about multiple alignements in a second TSV.

Tools

FROGS - FIND RAPIDLY OTU WITH GALAXY SOLUTION

FROGS pipeline

[FROGS Upload archive from your computer](#)

[FROGS Demultiplex reads](#)
 Split by samples the reads in function of inner barcode.

[FROGS Pre-process Step 1](#) in metagenomics analysis: denoising and dereplication.

[FROGS Clustering swarm](#)
 Step 2 in metagenomics analysis : clustering.

[FROGS Remove chimera](#) Step 3 in metagenomics analysis : Remove PCR chimera in each sample.

[FROGS Filters](#) Filters OTUs on several criteria.

[FROGS Affiliation OTU](#) Step 4 in metagenomics analysis : Taxonomic affiliation of each OTU's seed by RDPtools and BLAST

[FROGS BIOM to TSV](#) Converts a BIOM file in TSV file.

[FROGS Clusters stat](#) Process some metrics on clusters.

[FROGS Affiliations stat](#)
 Process some metrics on taxonomies.

[FROGS BIOM to std BIOM](#)
 Converts a FROGS BIOM in fully compatible BIOM.

[FROGS Abundance normalisation](#)

Exercise 6.1

5. Understand Blast affiliations - Cluster_2388 (affiliation from silva 123)

blast_subject	blast_evalue	blast_len	blast_perc_query_coverage	blast_perc_identity	blast_taxonomy
JN880417.1.1422	0.0	360	88.88	99.44	Bacteria;Planctomycetes;Planctomycetacia;Planctomycetales;Planctomycetaceae;Telmatocola;Telmatocola sphagniphila

Blast JN880417.1.1422 vs our OTU

OTU length : 405

Excellent blast but no matches at the beginning of OTU.

Telmatocola sphagniphila strain SP2 16S ribosomal RNA gene, partial sequence
 Sequence ID: [ref|NR_118328.1](#) Length: 1422 Number of Matches: 1

Range 1: 375 to 734 [GenBank](#) [Graphics](#) ▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
654 bits(354)	0.0	358/360(99%)	0/360(0%)	Plus/Plus
Query 46	CGCGTGCGCGATGAAGGCCTTCGGGTTGTAAGCGCGAAAGAGGTAATAAAGGGAAACCT	105		
Sbjct 375	CGCGTGCGCGATGAAGGCCTTCGGGTTGTAAGCGCGAAAGAGGSAATAAAGGGAAACTT	434		
Query 106	GATTGAACCTCAGTAAGCTCGGGCTAAGTTTGTGCCAGCAGCCGCGGTAAGACGAACCGA	165		
Sbjct 435	GATTGAACCTCAGTAAGCTCGGGCTAAGTTTGTGCCAGCAGCCGCGGTAAGACGAACCGA	494		
Query 166	GCGAACGTTGTTTCGGAATCACTGGGCATAAAGGGCGCGTAGGCGGGTTTCTAAGTCCGTG	225		
Sbjct 495	GCGAACGTTGTTTCGGAATCACTGGGCATAAAGGGCGCGTAGGCGGGTTTCTAAGTCCGTG	554		
Query 226	GTGAAATACTTCAGCTCAACTGGAGAAGTGCCTCGGATACTGGGAATCTCGAGTAATGTA	285		
Sbjct 555	GTGAAATACTTCAGCTCAACTGGAGAAGTGCCTCGGATACTGGGAATCTCGAGTAATGTA	614		
Query 286	GGGGCACGTGGAACGGCTGGTGGAGCGGTGAAATGCGTTGATATCAGTCGGAACCTCCGGT	345		
Sbjct 615	GGGGCACGTGGAACGGCTGGTGGAGCGGTGAAATGCGTTGATATCAGTCGGAACCTCCGGT	674		
Query 346	GGCGAAGGCGATGTGCTGGACATTTACTGACGCTGAGGCCGCGAAAGCCAGGGGAGCAAAC	405		
Sbjct 675	GGCGAAGGCGATGTGCTGGACATTTACTGACGCTGAGGCCGCGAAAGCCAGGGGAGCAAAC	734		

Telmatocola sphagniphila strain SP2 16S ribosomal RNA gene, partial sequence
 NCBI Reference Sequence: NR_118328.1
[FASTA](#) [Graphics](#)

[Go to:](#)

LOCUS	NR_118328	1422 bp	rRNA	linear	BCT 03-FEB-2015
DEFINITION	Telmatocola sphagniphila strain SP2 16S ribosomal RNA gene, partial sequence				
ACCESSION	NR_118328				
VERSION	NR_118328.1	GI:645321338			
DBLINK	Project: 33175				
	BioProject: PRJNA33175				
KEYWORDS	RefSeq.				
SOURCE	Telmatocola sphagniphila				
ORGANISM	Telmatocola sphagniphila				
	Bacteria; Planctomycetes; Planctomycetia; Planctomycetales; Planctomycetaceae.				
REFERENCE	1 (bases 1 to 1422)				
AUTHORS	Kulichevskaya, I.S., Serkebaeva, Y.M., Kim, Y., Rijpstra, W.I., Damste, J.S., Liesack, W. and Dedysh, S.N.				
TITLE	Telmatocola sphagniphila gen. nov., sp. nov., a novel dendriform planctomycete from northern wetlands				
JOURNAL	Front Microbiol 3, 146 (2012)				
PUBMED	22529844				
REMARK	Publication Status: Online-Only				
REFERENCE	2 (bases 1 to 1422)				
CONSRM	NCBI RefSeq Targeted Loci Project				
TITLE	Direct Submission				
JOURNAL	Submitted (28-APR-2014) National Center for Biotechnology Information, NIH, Bethesda, MD 20894, USA				
REFERENCE	3 (bases 1 to 1422)				
AUTHORS	Dedysh, S.N.				
TITLE	Direct Submission				
JOURNAL	Submitted (20-OCT-2011) Winogradsky Institute of Microbiology RAS, Prospect 60-Letya Otyabrya 7/2, Moscow 117312, Russia				
COMMENT	REVIEWED REFSEQ : This record has been curated by NCBI staff. The reference sequence is identical to JN880417.1-1422 .				

Blast columns

OTU_2 seed has a best BLAST hit with the reference sequence AJ496032.1.1410

The reference sequence taxonomic affiliation is this one.

#blast_taxonomy	blast_subject	blast_perc_identity	blast_perc_query_coverage	blast_evalue	blast_aln_length
Bacteria;Actinobacteria;Actinobacteria;Bifidobacteriales;Bifidobacteriaceae;Metascardovia;Multi-affiliation	multi-subject	100.0	100.0	0.0	411
Bacteria;Fibrobacteres;Fibrobacteria;Fibrobacterales;Fibrobacteraceae;Fibrobacter;Fibrobacter succinogenes	AJ496032.1.1410	100.0	100.0	0.0	419
Bacteria;Firmicutes;Bacilli;Bacillales;Staphylococcaceae;Nosocomiicoccus;Nosocomiicoccus ampullae	EU240886.1.1502	100.0	100.0	0.0	427
Bacteria;Proteobacteria;Gammaproteobacteria;Pseudomonadales;Moraxellaceae;Psychrobacter;Psychrobacter immobilis	U39399.1.1477	100.0	100.0	0.0	426
Bacteria;Thermotogae;Thermotogae;Thermotogales;Thermotogaceae;Petrotoga;Petrotoga miotherma	FR733705.1.1499	100.0	100.0	0.0	419
Bacteria;Proteobacteria;Alphaproteobacteria;Rhizobiales;Phyllobacteriaceae;Pseudahrensia;Pseudahrensia aquimaris	GU575117.1.1441	100.0	100.0	0.0	401
Bacteria;Bacteroidetes;Cytophagia;Cytophagales;Cytophagaceae;Persicitalea;Persicitalea jodogahamensis	multi-subject	100.0	100.0	0.0	421
Bacteria;Proteobacteria;Deltaproteobacteria;Bdellovibrionales;Bdellovibrionaceae;Bdellovibrio;Multi-affiliation	multi-subject	100.0	100.0	0.0	404

Convert to TSV

FROGS BIOM to TSV ✕

Abundance file

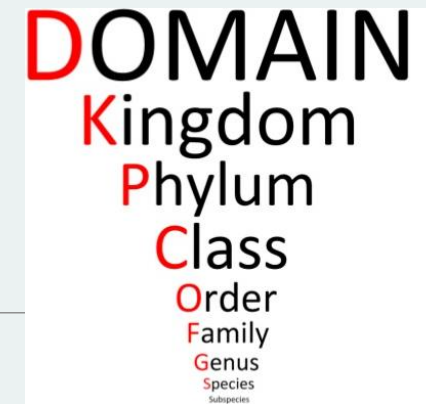
Sequences file

tsv_file (tabular)

multi_affi_file (tabular)

Evaluation variables of BLAST

Focus on “Multi-”



(affiliation from silva 123)

Observe line of Cluster 1 inside abundance.tsv and multi_hit.tsv files, what do you conclude ?

#blast_taxonomy	blast_subject	blast_perc_identity	blast_perc_query_coverage	blast_evalue	blast_aln_length
Bacteria;Actinobacteria;Actinobacteria;Bifidobacteriales;Bifidobacteriaceae;Metascardovia;Multi-affiliation	multi-subject	100.0	100.0	0.0	411
Bacteria;Fibrobacteres;Fibrobacteria;Fibrobacterales;Fibrobacteraceae;Fibrobacter;Fibrobacter succinogenes	AJ496032.1.1410	100.0	100.0	0.0	419
Bacteria;Firmicutes;Bacilli;Bacillales;Staphylococcaceae;Nosocomiicoccus;Nosocomiicoccus ampullae	EU240886.1.1502	100.0	100.0	0.0	427
Bacteria;Proteobacteria;Gammaproteobacteria;Pseudomonadales;Moraxellaceae;Psychrobacter;Psychrobacter immobilis	U39399.1.1477	100.0	100.0	0.0	426
Bacteria;Thermotogae;Thermotogae;Thermotogales;Thermotogaceae;Petrotoga;Petrotoga miotherma	FR733705.1.1499	100.0	100.0	0.0	419
Bacteria;Proteobacteria;Alphaproteobacteria;Rhizobiales;Phyllobacteriaceae;Pseudahrensia;Pseudahrensia aquimaris	GU575117.1.1441	100.0	100.0	0.0	401
Bacteria;Bacteroidetes;Cytophagia;Cytophagales;Cytophagaceae;Persicitalea;Persicitalea jodogahamensis	multi-subject	100.0	100.0	0.0	421
Bacteria;Proteobacteria;Deltaproteobacteria;Bdellovibrionales;Bdellovibrionaceae;Bdellovibrio;Multi-affiliation	multi-subject	100.0	100.0	0.0	404

Cluster_1 has 5 identical blast hits, with different taxonomies as the species level

Focus on “Multi-”

(affiliation from silva 123)

Observe line of Cluster 11 inside abundance.tsv and multi_hit.tsv files, what do you conclude ?

Bacteria;Proteobacteria;Alphaproteobacteria;Caulobacterales;Hyphomonadaceae;Henriciella;Henriciella marina	multi-subject	100.0	100.0
--	---------------	-------	-------



Cluster_11 has 2 identical blast hits, with identical species but with different strains (strains are not written in our data)

Focus on “Multi-”

(affiliation from silva 123)

Observe line of Cluster 43 inside abundance.tsv and multi_hit.tsv files, what do you conclude ?

Bacteria;Firmicutes;Negativicutes;Selenomonadales;Veillonellaceae;Multi-affiliation;Multi-affiliation	multi-subject	99.3	100.0
Cluster_43	Bacteria;Firmicutes;Negativicutes;Selenomonadales;Veillonellaceae;Selenomonas 3;unknown species		JQ447821.1.1420
Cluster_43	Bacteria;Firmicutes;Negativicutes;Selenomonadales;Veillonellaceae;Centipeda;Centipeda periodontii		AJ010963.1.1494

Cluster_43 has 2 identical blast hits, with different taxonomies at the genus level

Back on Blast parameters

#blast_taxonomy	blast_subject	blast_perc_identity	blast_perc_query_coverage	blast_evalue	blast_aln_length
Bacteria;Actinobacteria;Actinobacteria;Bifidobacteriales;Bifidobacteriaceae;Metascardovia;Multi-affiliation	multi-subject	100.0	100.0	0.0	411
Bacteria;Fibrobacteres;Fibrobacteria;Fibrobacterales;Fibrobacteraceae;Fibrobacter;Fibrobacter succinogenes	AJ496032.1.1410	100.0	100.0	0.0	419
Bacteria;Firmicutes;Bacilli;Bacillales;Staphylococcaceae;Nosocomiicoccus;Nosocomiicoccus ampullae	EU240886.1.1502	100.0	100.0	0.0	427
Bacteria;Proteobacteria;Gammaproteobacteria;Pseudomonadales;Moraxellaceae;Psychrobacter;Psychrobacter immobilis	U39399.1.1477	100.0	100.0	0.0	426
Bacteria;Thermotogae;Thermotogae;Thermotogales;Thermotogaceae;Petrotoga;Petrotoga miotherma	FR733705.1.1499	100.0	100.0	0.0	419
Bacteria;Proteobacteria;Alphaproteobacteria;Rhizobiales;Phyllobacteriaceae;Pseudahrensia;Pseudahrensia aquimaris	GU575117.1.1441	100.0	100.0	0.0	401
Bacteria;Bacteroidetes;Cytophagia;Cytophagales;Cytophagaceae;Persicitalea;Persicitalea jodogahamensis	multi-subject	100.0	100.0	0.0	421
Bacteria;Proteobacteria;Deltaproteobacteria;Bdellovibrionales;Bdellovibrionaceae;Bdellovibrio;Multi-affiliation	multi-subject	100.0	100.0	0.0	404

Evaluation variables of BLAST

Blast variables : e-value

The Expect value (E) is a parameter that describes the number of hits one can "expect" to see by chance when searching a database of a particular size.

The lower the E-value, or the closer it is to zero, the more "significant" the match is.

Blast variables : blast_perc_identity

Identity percentage between the Query (OTU) and the subject in the alignment
(length subject = 1455 bases)

Score	Expect	Identities	Gaps	Strand
760 bits(411)	0.0	411/411(100%)	0/411(0%)	Plus/Plus
Query 1	TGGGGAATATTGCACAATGGGGGGAACCCTGATGCAGCGACGCCGCGTGCGGGATGACGG	60		
Sbjct 331	TGGGGAATATTGCACAATGGGGGGAACCCTGATGCAGCGACGCCGCGTGCGGGATGACGG	390		
Query 61	CCTTCGGGTTGTAAACCGCTTTTAAATGGGAGCAAGCAGTTTTACTGTGAGTGTACTTTT	120		
Sbjct 391	CCTTCGGGTTGTAAACCGCTTTTAAATGGGAGCAAGCAGTTTTACTGTGAGTGTACTTTT	450		
Query 121	TGAATAAGCACCCGGCTAACTACGTGCCAGCAGCCCGGTAATACGTAGGGTGCAAGCGTT	180		
Sbjct 451	TGAATAAGCACCCGGCTAACTACGTGCCAGCAGCCCGGTAATACGTAGGGTGCAAGCGTT	510		
Query 181	GTCCGGAATTATTGGGCGTAAAGAGCTCGTAGGCGGTTTGTCCGCTCTGGTGTGAAAGTC	240		
Sbjct 511	GTCCGGAATTATTGGGCGTAAAGAGCTCGTAGGCGGTTTGTCCGCTCTGGTGTGAAAGTC	570		
Query 241	CATCGCTTAACGGTGGATTTGCGCTGGGTACGGCAGGCTAGAGTGTAGTAGGGGAGACT	300		
Sbjct 571	CATCGCTTAACGGTGGATTTGCGCTGGGTACGGCAGGCTAGAGTGTAGTAGGGGAGACT	630		
Query 301	GGAATTCGGGTGTAAACGGTGGAAATGTGTAGATATCGGGAAGAACACCAATGGCGAAGGC	360		
Sbjct 631	GGAATTCGGGTGTAAACGGTGGAAATGTGTAGATATCGGGAAGAACACCAATGGCGAAGGC	690		
Query 361	AGGTCTCTGGGCTATGACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAAC	411		
Sbjct 691	AGGTCTCTGGGCTATGACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAAC	741		

Query length = 411
Alignment length = 411
0 mismatch
-> 100% identity

Blast variables :

blast_perc_identity

Identity percentage between the Query (OTU) and the subject in the alignment
(length subject = 1455 bases)

Score	Expect	Identities	Gaps	Strand
614 bits(332)	5e-172	385/411(94%)	5/411(1%)	Plus/Plus
Query 1	TGGGGAATATTGCACAATGGGGGGAACCTGATGCAGCGACGCCGCGTGCGGGATGACGG	60		
Sbjct 140728	TGGGGAATATTGCACAATGGGCGAAAGCCTGATGCAGCGACGCCGCGTGCGGGATGACGG	140787		
Query 61	CCTTCGGGTTGTAAACCGCTTTTAAATTGGGAGCAAGCAGTTTACTGTGAGTGTACTTTT	120		
Sbjct 140788	CCTTCGGGTTGTAAACCGCTTTTGAATTGGGAGCAAGC-G----AGAGTGTGTACCTTT	140842		
Query 121	TGAATAAGCACCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGTGCAAGCGTT	180		
Sbjct 140843	CGAATAAGCACCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGTGCAAGCGTT	140902		
Query 181	GTCCGGAATTATTGGGCGTAAAGAGCTCGTAGGCGGTTTGTTCGCGTCTGGTGTGAAAGTC	240		
Sbjct 140903	ATCCGGAATTATTGGGCGTAAAGRGCTCGTAGGCGGTTCTGTCGCGTCTGGTGTGAAAGTC	140962		
Query 241	CATCGCTTAACGGTGGATTTGCGCTGGGTACGGGCAGGCTAGAGTGTAGTAGGGGAGACT	300		
Sbjct 140963	CATCGCTTAACGGTGGATCTGCGCCGGGTACGGGCAGGCTAGAGTGTAGTAGGGGAGACT	141022		
Query 301	GGAATCCCGGTGTAAACGGTGGAAATGTGTAGATATCGGGAAGAACCAATGGCGAAGGC	360		
Sbjct 141023	GGAATCCCGGTGTAAACGGTGGAAATGTGTAGATATCGGGAAGAACCAATGGCGAAGGC	141082		
Query 361	AGGTCTCTGGGCTATGACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAAC	411		
Sbjct 141083	AGGTCTCTGGGCCGTACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAAC	141133		

Query length = 411
Alignment length = 411
26 mismatches (gaps included)
-> 94% identity

Blast variables : blast_perc_query_coverage

Coverage percentage of alignment on query (OTU)

Score	Expect	Identities	Gaps	Strand
760 bits(411)	0.0	411/411(100%)	0/411(0%)	Plus/Plus
Query 1	TGGGGAATATTGCACAATGGGGGGAACCTGATGCAGCGACGCCGCGTGCGGGATGACGG	60		
Sbjct 331	TGGGGAATATTGCACAATGGGGGGAACCTGATGCAGCGACGCCGCGTGCGGGATGACGG	390		
Query 61	CCTTCGGGTTGTAAACCGCTTTTAAATGGGAGCAAGCAGTTTACTGTGAGTGTACTTTT	120		
Sbjct 391	CCTTCGGGTTGTAAACCGCTTTTAAATGGGAGCAAGCAGTTTACTGTGAGTGTACTTTT	450		
Query 121	TGAATAAGCACCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGTGCAAGCGTT	180		
Sbjct 451	TGAATAAGCACCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGTGCAAGCGTT	510		
Query 181	GTCCGGAATTATTGGGCGTAAAGAGCTCGTAGGCGGTTTGTCCGCTCTGGTGTGAAAGTC	240		
Sbjct 511	GTCCGGAATTATTGGGCGTAAAGAGCTCGTAGGCGGTTTGTCCGCTCTGGTGTGAAAGTC	570		
Query 241	CATCGCTTAACGGTGGATTTGCGCTGGGTACGGGCAGGCTAGAGTGTAGTAGGGGAGACT	300		
Sbjct 571	CATCGCTTAACGGTGGATTTGCGCTGGGTACGGGCAGGCTAGAGTGTAGTAGGGGAGACT	630		
Query 301	GGAATTCGGGTGTAACGGTGGAAATGTGTAGATATCGGGAAGAACACCAATGGCGAAGGC	360		
Sbjct 631	GGAATTCGGGTGTAACGGTGGAAATGTGTAGATATCGGGAAGAACACCAATGGCGAAGGC	690		
Query 361	AGGTCTCTGGGCTATGACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAAC	411		
Sbjct 691	AGGTCTCTGGGCTATGACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAAC	741		

Query length = 411
100% coverage

Blast variables : blast-length

Length of alignment between the OTUs = “Query” and “subject” sequence of database

	Coverage %	Identity %	Length alignment
OTU1	100	98	400
OTU2	100	98	500



More mismatches/gaps

FROGS Affiliation OTU ✕

- OTU seed sequence
- Abundance file
- biom_affiliation (biom1) 🔄
- summary (html) 🔄

Affiliation

FROGS Affiliation OTU Step 4 in metagenomics analysis : Taxonomic affiliation of each OTU's seed by RDPtools and BLAST (Galaxy Version 0.8.0) Options

Using reference database
 silva123 16S
 Select reference from the list

Also perform RDP assignation?
 Yes No

Taxonomy affiliation will be perform thanks to Blast. This option allow you to perform it also with RDP classifier (default No)

OTU seed sequence
 17: FROGS Filters: sequences.fasta
 OTU sequences (format: fasta).

Abundance file
 18: FROGS Filters: abundance.biom
 OTU abundances (format: BIOM).

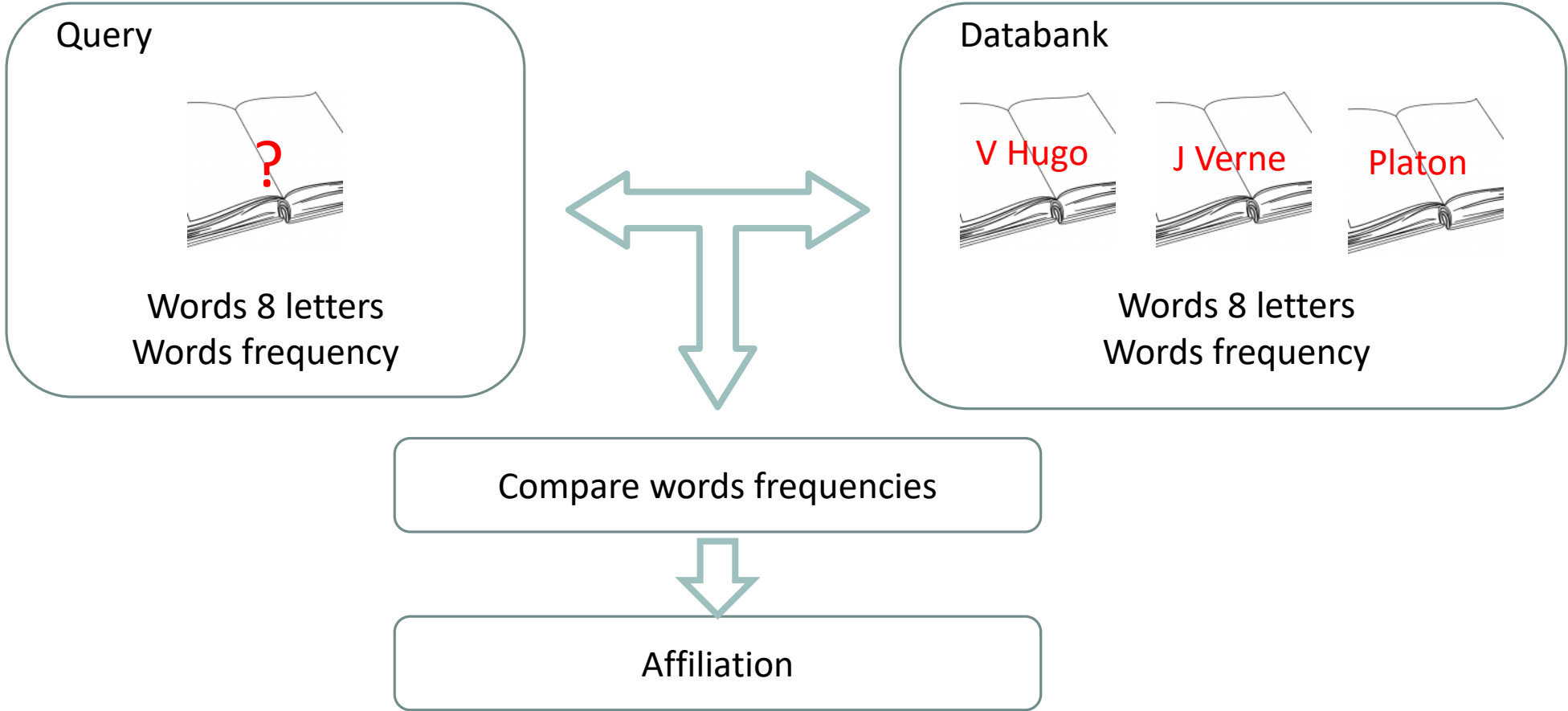
Execute

Optional and not in our guideline

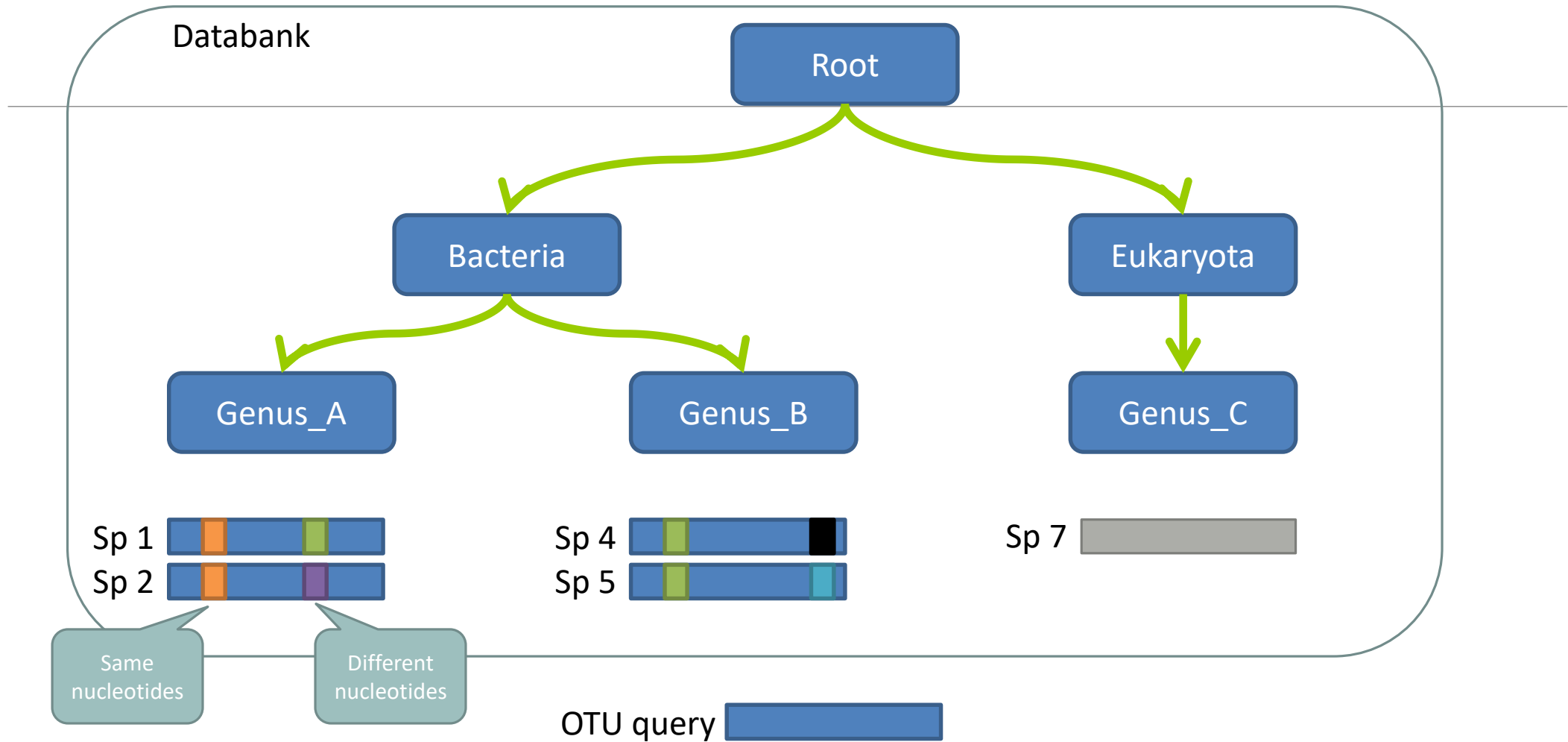
Who have already used RDP previously ?



How works RDP ?

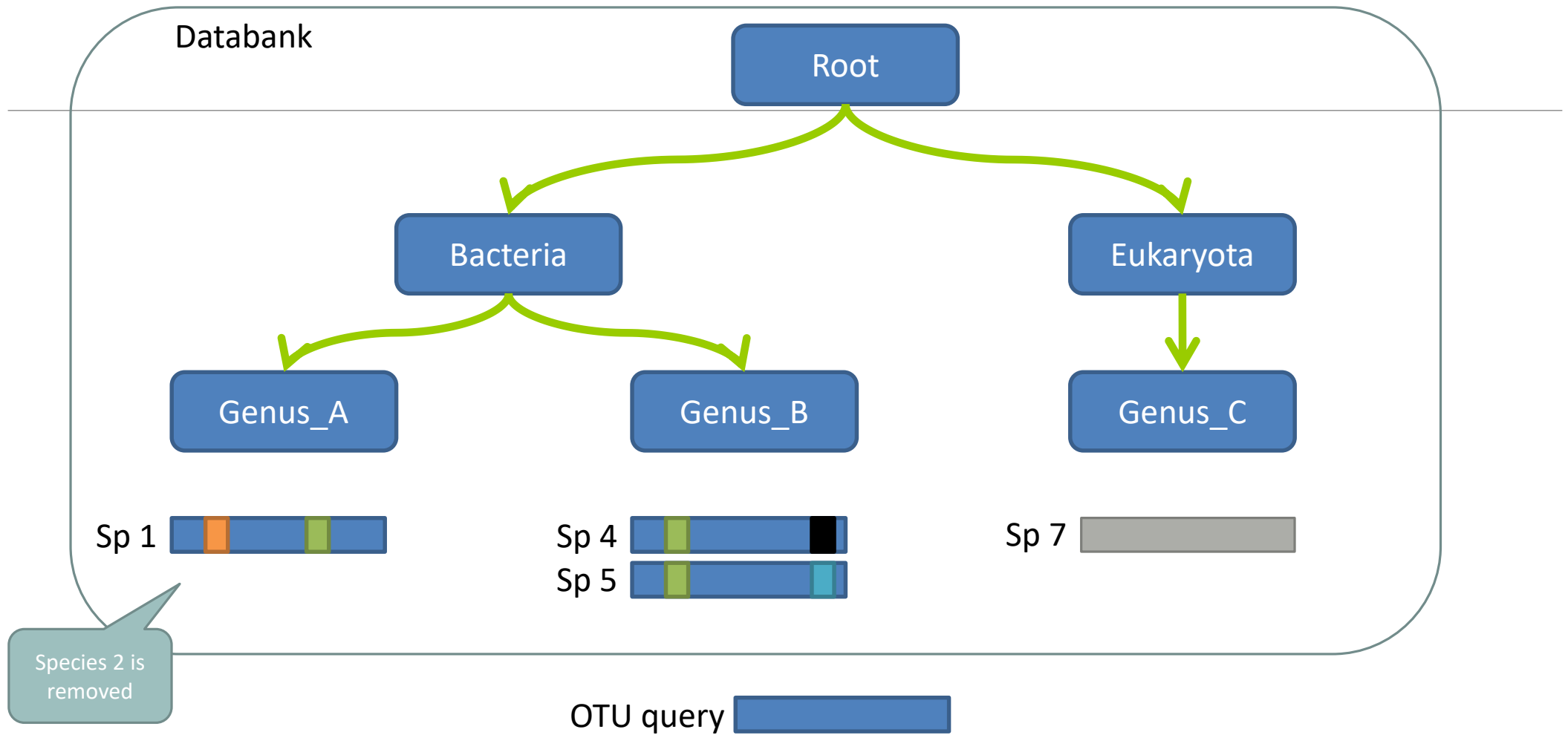


How works RDP ?



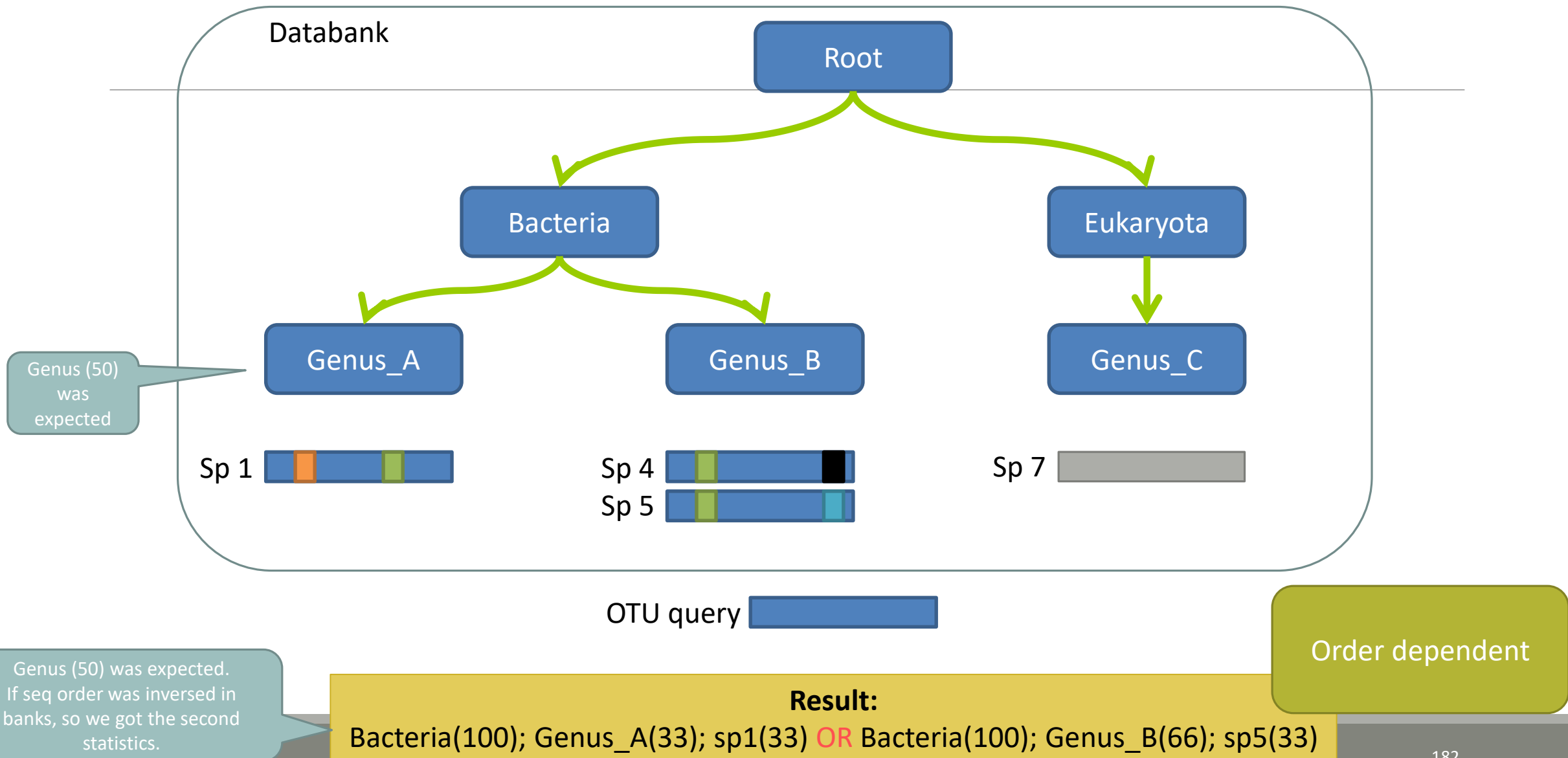
Result:
Bacteria(100) ; Genus_A(50) ; Sp1(25)

The dysfunctions of RDP ?

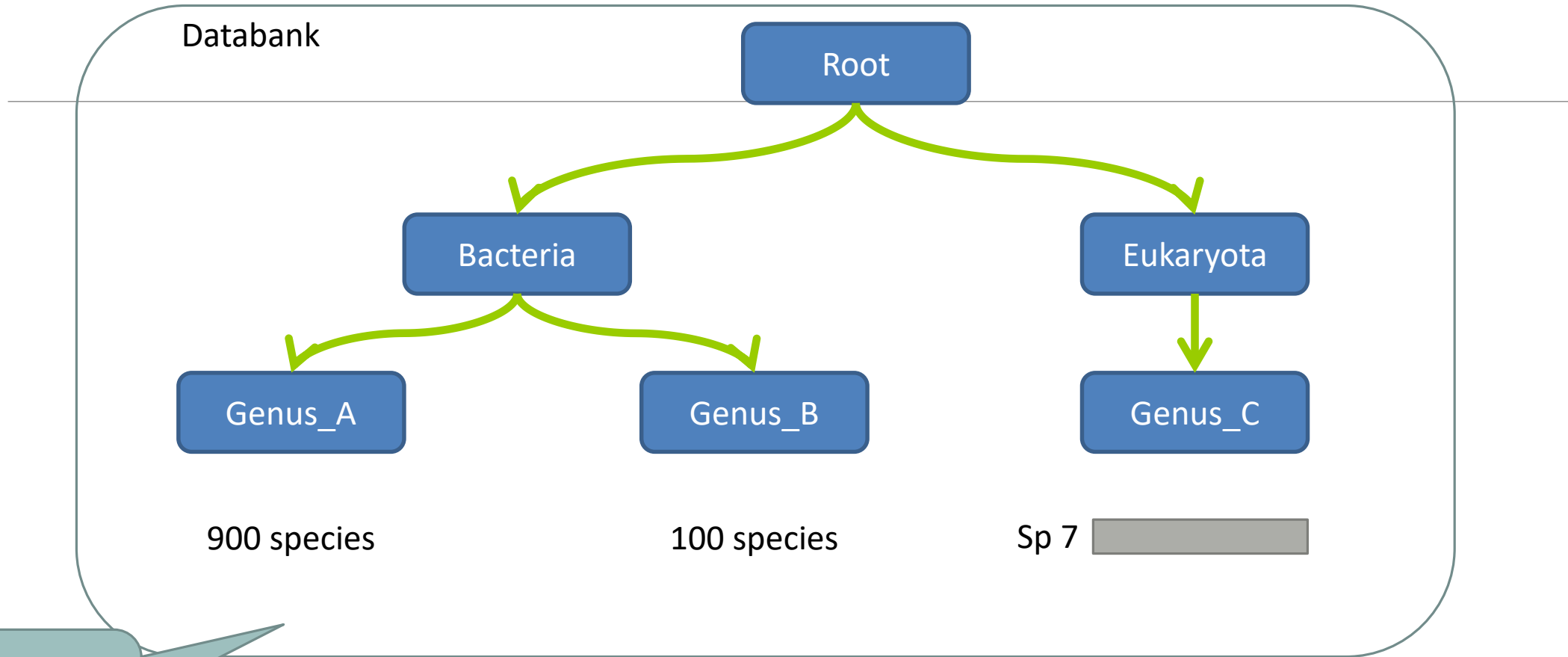


Result:
?

The dysfunctions of RDP n°1 ?



The dysfunctions of RDP n°2 ?



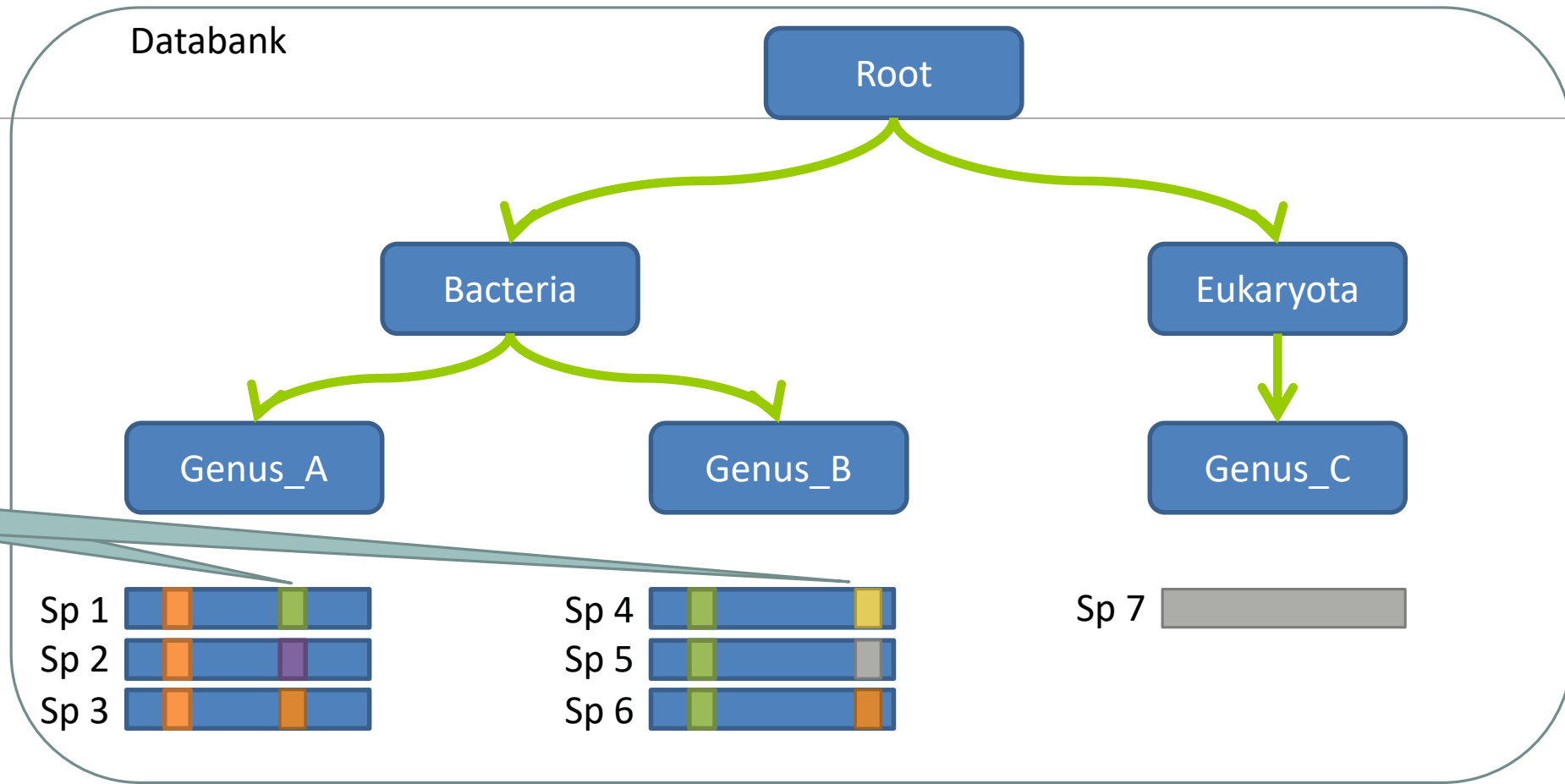
Many species in one genus and little in the other:
So, RDP can give very different results

Result:

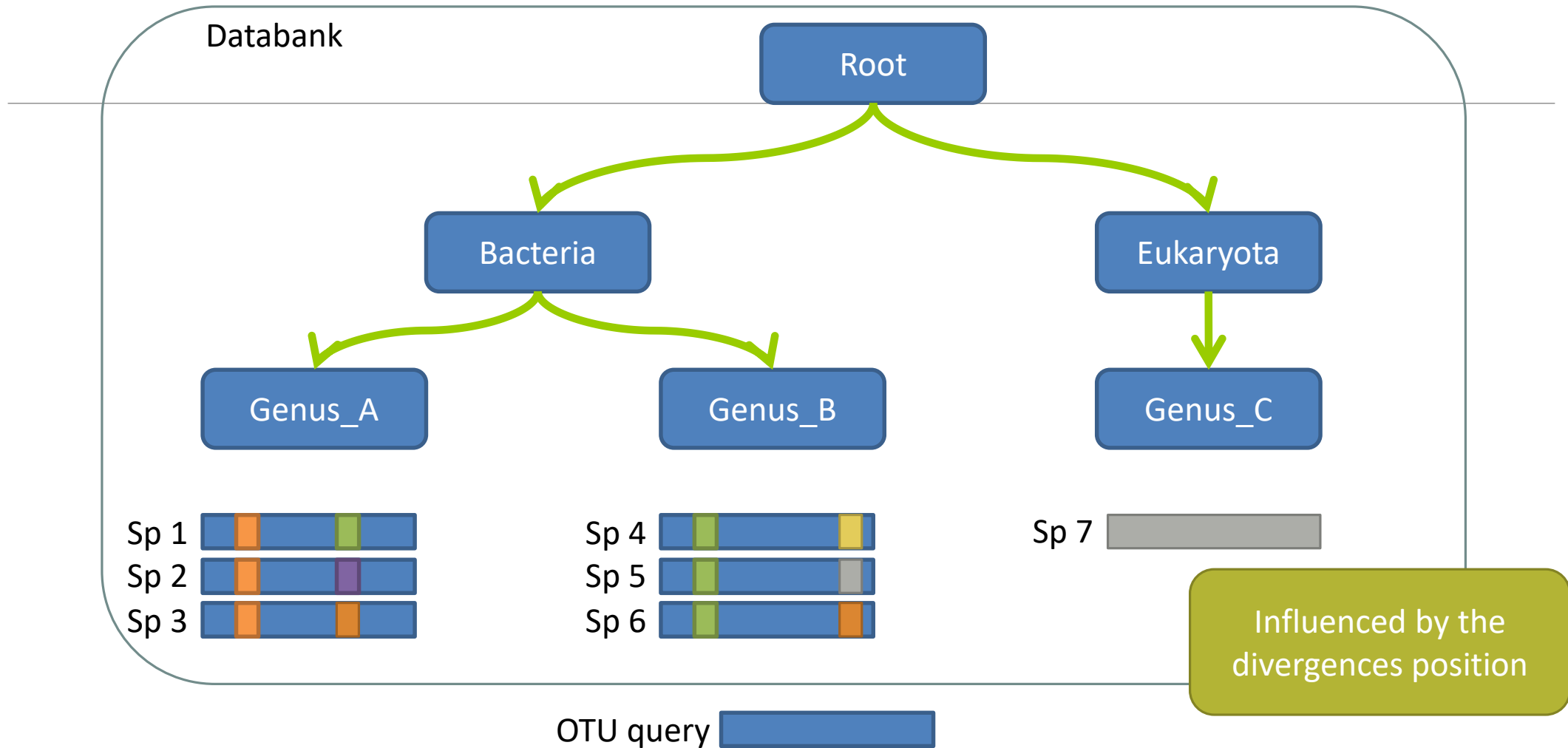
Bacteria(100); Genus_A(90); spX(0.1) **OR** Bacteria(100); Genus_B(10); spX(0.1)

Influenced by heterogeneity in last ranks

The dysfunctions of RDP n°3 ?

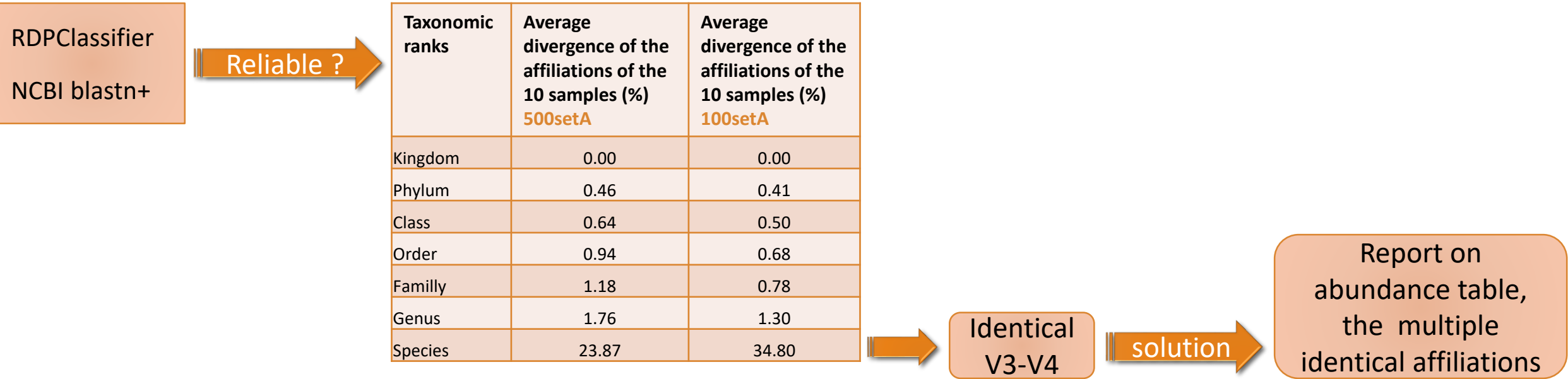


The dysfunctions of RDP n°3 ?



Si le mismatch se fait sur un mot très "significatif" dans le profil de k-mers, RDP ne tombera que rarement sur l'espèce lors du bootstrap. Avec une même distance d'édition (2 mismatches) on peut donc avoir une grande différence de bootstrap pour peu que le mot affecté soit important dans le profil.

Divergence on the composition of microbial communities at the different taxonomic ranks



Only one best hit

Taxonomic ranks	Average divergence of the affiliations of the 10 samples (%) 500setA	Average divergence of the affiliations of the 10 samples (%) 100setA
Kingdom	0.00	0.00
Phylum	0.46	0.41
Class	0.64	0.50
Order	0.94	0.68
Familly	1.18	0.78
Genus	1.76	1.30
Species	23.87	34.80

Multiple best hit

Taxonomic ranks	Median divergence of the affiliations of the 10 samples (%) 500setA	Median divergence of the affiliations of the 10 samples (%) 100setA
Kingdom	0.00	0.00
Phylum	0.46	0.41
Class	0.64	0.50
Order	0.93	0.68
Familly	1.17	0.78
Genus	1.60	1.00
Species	6.63	5.75

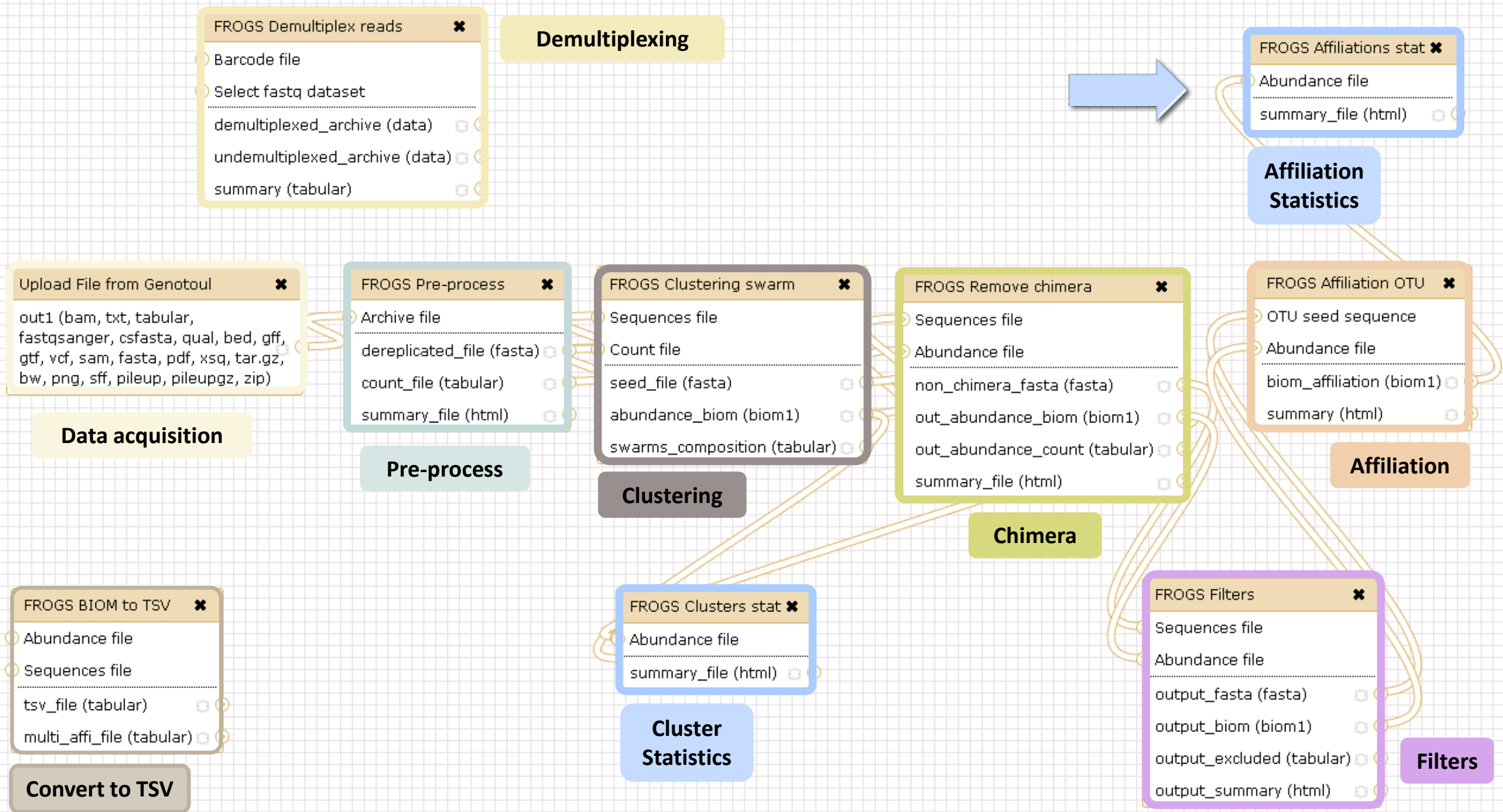
With the FROGS guideline

Taxonomic ranks	Median divergence of the affiliations of the 10 samples (%) 500setA filter: 0.005% - 505 OTUs	Median divergence of the affiliations of the 10 samples (%) 100setA filter: 0.005% - 100 OTUs
Kingdom	0.00	0.00
Phylum	0.38	0.38
Class	0.57	0.48
Order	0.81	0.64
Familly	1.08	0.74
Genus	1.43	0.76
Species	1.53	0.78

Careful: Multi hit blast table is non exhaustive !

- Chimera (multiple affiliation)
- V3V4 included in others
- Missed primers on some 16S during database building

Affiliation Stat



FROGS Affiliations stat Process some metrics on taxonomies. (Galaxy Version 1.1.0) Options

Abundance file
 22: FROGS Affiliation OTU: affiliation.biom
 OTUs abundances and affiliations (format: BIOM).

Rarefaction ranks
 Class Order Family Genus Species
 The ranks that will be evaluated in rarefaction. Each rank is separated by one space.

Affiliation processed
 FROGS blast
 Select the type of affiliation processed. If your affiliation has been processed with an external tool: use 'Custom'.

Execute

FROGS Affiliations stat Process some metrics on taxonomies. (Galaxy Version 1.1.0) Options

Abundance file
 22: FROGS Affiliation OTU: affiliation.biom
 OTUs abundances and affiliations (format: BIOM).

Rarefaction ranks
 Class Order Family Genus Species
 The ranks that will be evaluated in rarefaction. Each rank is separated by one space.

Affiliation processed
 FROGS rdp
 Select the type of affiliation processed. If your affiliation has been processed with an external tool: use 'Custom'.

Execute



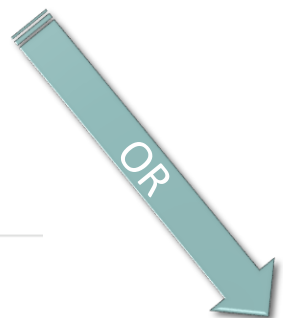
Taxonomy distribution

Alignment distribution



Taxonomy distribution

Bootstrap distribution



FROGS Affiliations stat Process some metrics on taxonomies. (Galaxy Version 1.1.0) Options

Abundance file
 22: FROGS Affiliation OTU: affiliation.biom
 OTUs abundances and affiliations (format: BIOM).

Rarefaction ranks
 Class Order Family Genus Species
 The ranks that will be evaluated in rarefaction. Each rank is separated by one space.

Affiliation processed
 Custom
 Select the type of affiliation processed. If your affiliation has been processed with an external tool: use 'Custom'.

Taxonomic ranks
 Domain Phylum Class Order Family Genus Species
 The ordered taxonomic ranks levels stored in BIOM. Each rank is separated by one space.

Taxonomy tag
 taxonomy
 The metadata title in BIOM for the taxonomy.

Bootstrap tag
 The metadata title in BIOM for the taxonomy bootstrap.

Identity tag
 The metadata tag used in BIOM file to store the alignment identity.

Coverage tag
 The metadata tag used in BIOM file to store the alignment OTUs coverage.

Execute

Exercise 6.2

FROGS Affiliations stat (version 1.1.0)

Abundance file:
17: FROGS Affiliation OTU: affiliation.biom
OTUs abundances and affiliations (format: BIOM).

Rarefaction ranks:
Class Order Family Genus Species
The ranks that will be evaluated in rarefaction. Each rank is separated by one space.

Affiliation processed:
FROGS blast
Select the type of affiliation processed. If your affiliation has been processed with an external tool: use 'Custom'.

Execute

FROGS Affiliations stat (version 1.1.0)

Abundance file:
17: FROGS Affiliation OTU: affiliation.biom
OTUs abundances and affiliations (format: BIOM).

Rarefaction ranks:
Class Order Family Genus Species
The ranks that will be evaluated in rarefaction. Each rank is separated by one space.

Affiliation processed:
FROGS rdp **Is it adequate on our data ? Why ?**
Select the type of affiliation processed. If your affiliation has been processed with an external tool: use 'Custom'.

Execute

23: FROGS Affiliations stat: summary.html

Exercise 6.2

→ objectives :

understand rarefaction curve and sunburst

1. Explore the [Affiliation stat](#) results on FROGS blast affiliation.
2. What kind of graphs can you generate? What do they mean?

Tools

RADseq STACKS

RADseq STACKS

METHYLATION - BISULFITE

Bisulfite BISMARK

DEEPTOOLS

deepTools

FROGS - FIND RAPIDLY OTU WITH GALAXY SOLUTION

FROGS pipeline

FROGS Upload archive from your computer

FROGS Demultiplex reads Split by samples the reads in function of inner barcode.

FROGS Pre-process Step 1 in metagenomics analysis: denoising and dereplication.

FROGS Clustering swarm Step 2 in metagenomics analysis : clustering.

FROGS Remove chimera Step 3 in metagenomics analysis : Remove PCR chimera in each sample.

FROGS Filters Filters OTUs on several criteria.

FROGS Affiliation OTU Step 4 in metagenomics analysis : Taxonomic affiliation of each OTU's seed by RDPtools and BLAST

FROGS BIOM to TSV Converts a BIOM file in TSV file.

FROGS Clusters stat Process some metrics on clusters.

FROGS Affiliations stat Process some metrics on taxonomies.

FROGS BIOM to std BIOM Converts a FROGS BIOM in

Taxonomy distribution Alignment distribution

Display global distribution

CSV

Show 10 entries

Search:

Taxonomies by sample

<input type="checkbox"/> Samples	Nb domain	Nb phylum	Nb class	Nb order	Nb family	Nb genus	Nb species	Nb sequences
<input checked="" type="checkbox"/> 500taxas_With_Error_Power_Law-01-reads	1	29	59	129	243	491	492	81,572
<input checked="" type="checkbox"/> 500taxas_With_Error_Power_Law-02-reads	1	29	59	130	243	491	492	82,466
<input checked="" type="checkbox"/> 500taxas_With_Error_Power_Law-03-reads	1	29	59	130	243	491	493	82,159
<input type="checkbox"/> 500taxas_With_Error_Power_Law-04-reads	1	29	59	130	243	491	492	81,985
<input type="checkbox"/> 500taxas_With_Error_Power_Law-05-reads	1	29	59	130	241	487	488	82,039
<input type="checkbox"/> 500taxas_With_Error_Power_Law-06-reads	1	29	59	130	244	493	494	81,758
<input type="checkbox"/> 500taxas_With_Error_Power_Law-07-reads	1	29	59	130	244	491	492	81,714
<input type="checkbox"/> 500taxas_With_Error_Power_Law-08-reads	1	29	58	129	243	493	494	82,255
<input type="checkbox"/> 500taxas_With_Error_Power_Law-09-reads	1	29	59	130	244	493	494	82,113
<input type="checkbox"/> 500taxas_With_Error_Power_Law-10-reads	1	29	58	128	240	487	489	82,300



With selection: Class

Showing 1 to 10 of 10 entries

Previous 1 Next

History

imported: 500WEPL_setA
451.3 MB

106: FROGS Clusters stat summary.html

105: report_download

103: Vsearch Clusters stat

102: FROGS Affiliations stat summary.html
299.1 KB
format: html, database: ?
Application Software:
affiliations_stat.py (version: 1.1.0)
Command: /usr/local/bioinfo
/src/galaxy-dev/galaxy-dist/tools
/FROGS/tools/affiliations_stat.py
--input-biom /galaxydata/database
/files/054/dataset_54829.dat
--output-file /work/galaxy-dev/data

HTML file

101: swarm cluster stat

100: FROGS BIOM to std BIOM: blast metadata.tsv

99: FROGS BIOM to std BIOM: abundance.biom

98: FROGS BIOM to TSV: multi_hits.tsv

97: FROGS BIOM to TSV: abundance.tsv

96: FROGS Affiliations stat summary.html
295.0 KB
format: html, database: ?
Application Software:
affiliations_stat.py (version: 1.1.0)
Command: /usr/local/bioinfo

Tools

[FROGS Demultiplex reads](#)
Split by samples the reads in function of inner barcode.

[FROGS Pre-process](#) Step 1 in metagenomics analysis: denoising and dereplication.

[FROGS Clustering swarm](#)
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[FROGS Affiliation OTU](#) Step 4 in metagenomics analysis : Taxonomic affiliation of each OTU's seed by RDPtools and BLAST

[FROGS BIOM to TSV](#) Converts a BIOM file in TSV file.

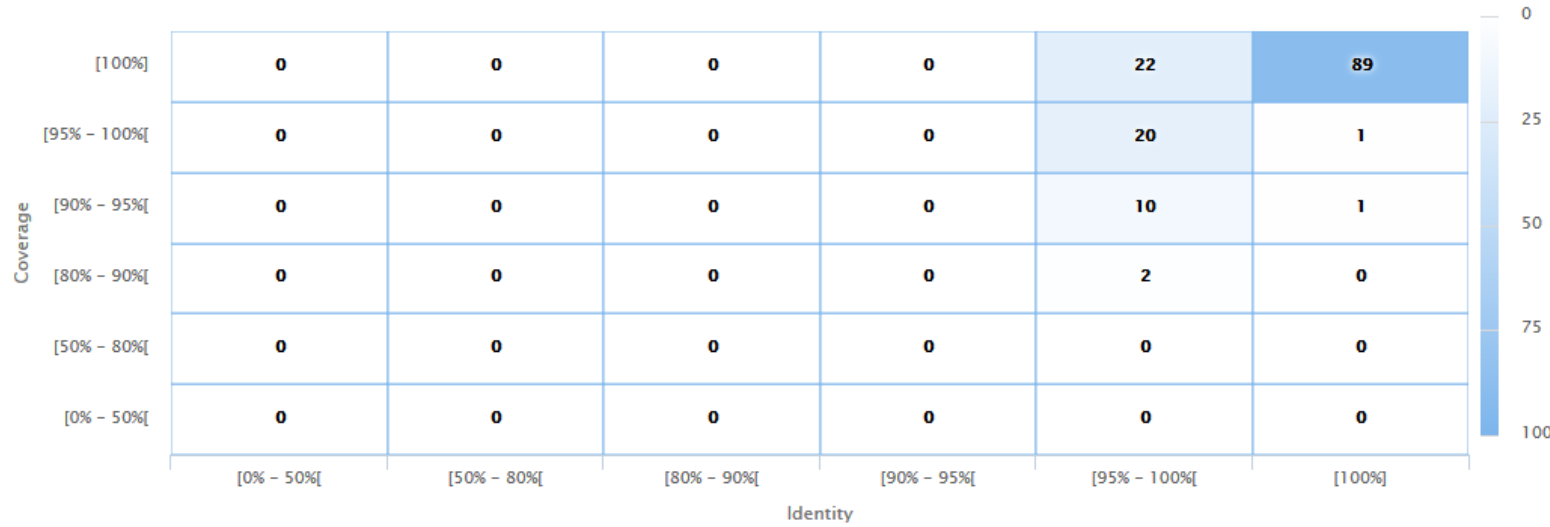
[FROGS Clusters stat](#) Process some metrics on clusters.

[FROGS Affiliations stat](#)
Process some metrics on taxonomies.

Taxonomy distribution

Alignment distribution

Number of OTUs among their alignment results



by OTUs

by sequences

History

Formation 9samples

20.3 MB

[21: FROGS BIOM to TSV: multi_hits.tsv](#)

[20: FROGS BIOM to TSV: abundance.tsv](#)

[19: FROGS Affiliations stat: summary.html](#)

230.0 KB

format: html, database: ?
Application Software:
affiliations_stat.py (version: 1.1.0) Command: /usr/local/bioinfo/src/galaxy-dev/galaxy-dist/tools/FROGS/tools/affiliations_stat.py --input-biom /galaxydata/database/files/060/dataset_60522.dat --output-file /work/galaxy-dev/data

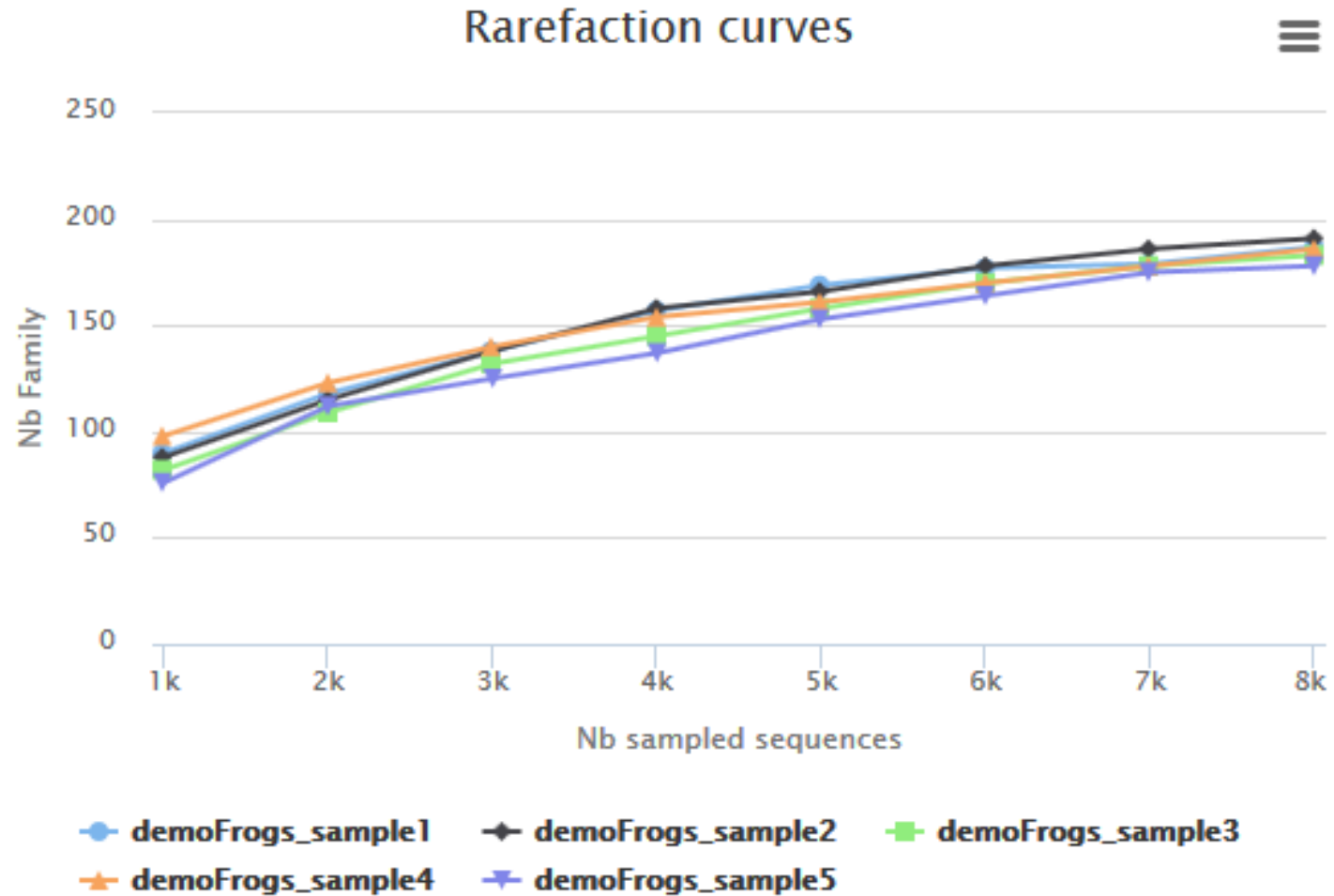
HTML file

[18: FROGS Affiliation OTU: report.html](#)

Available only after
AFFILIATION TOOL

Samples size ~8500
sequences

Rarefaction



The curve continues
to rise

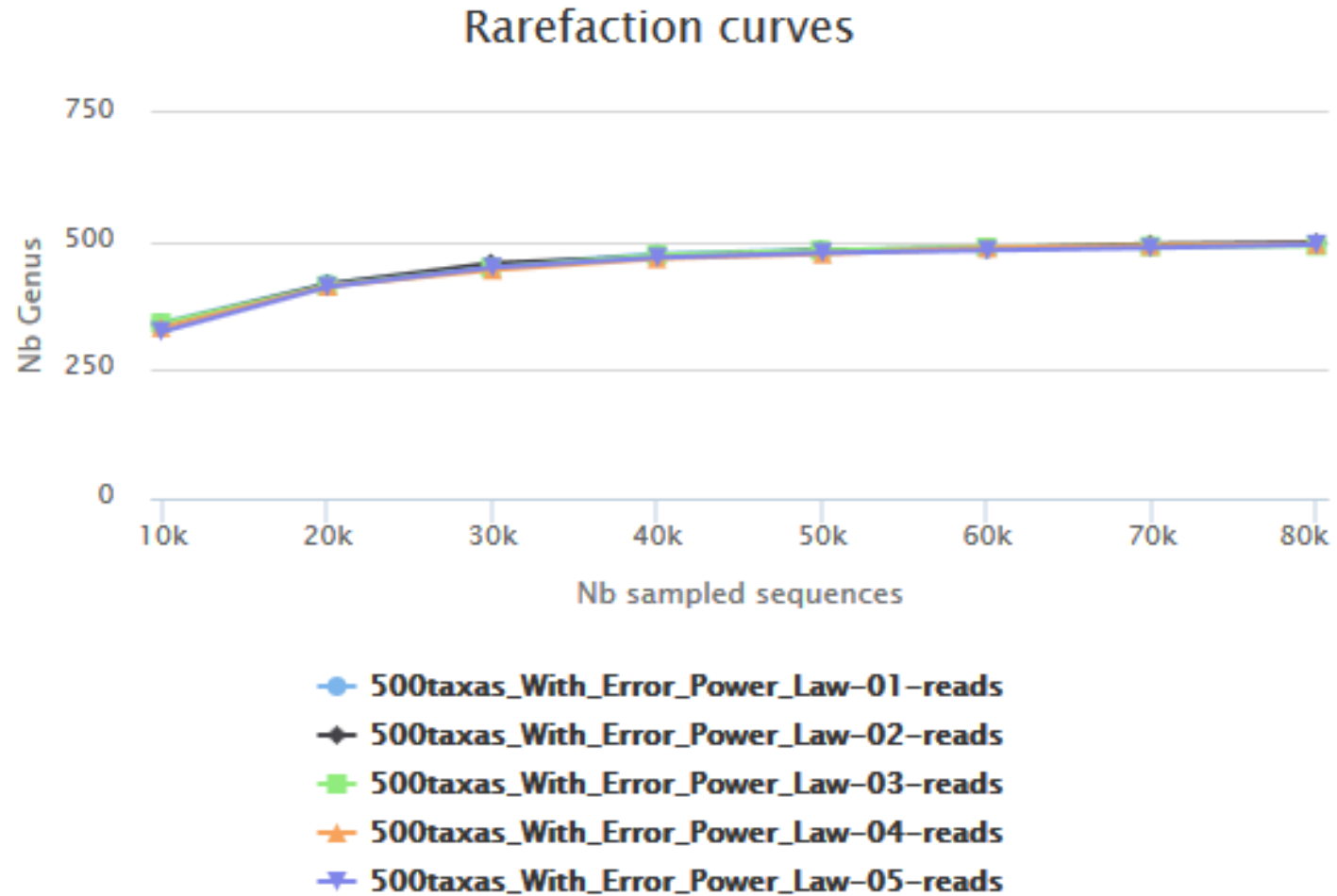
The number of
sequences per
sample is not large
enough to cover all
of the bacterial
families

Rarefaction tab

Available only after
AFFILIATION TOOL

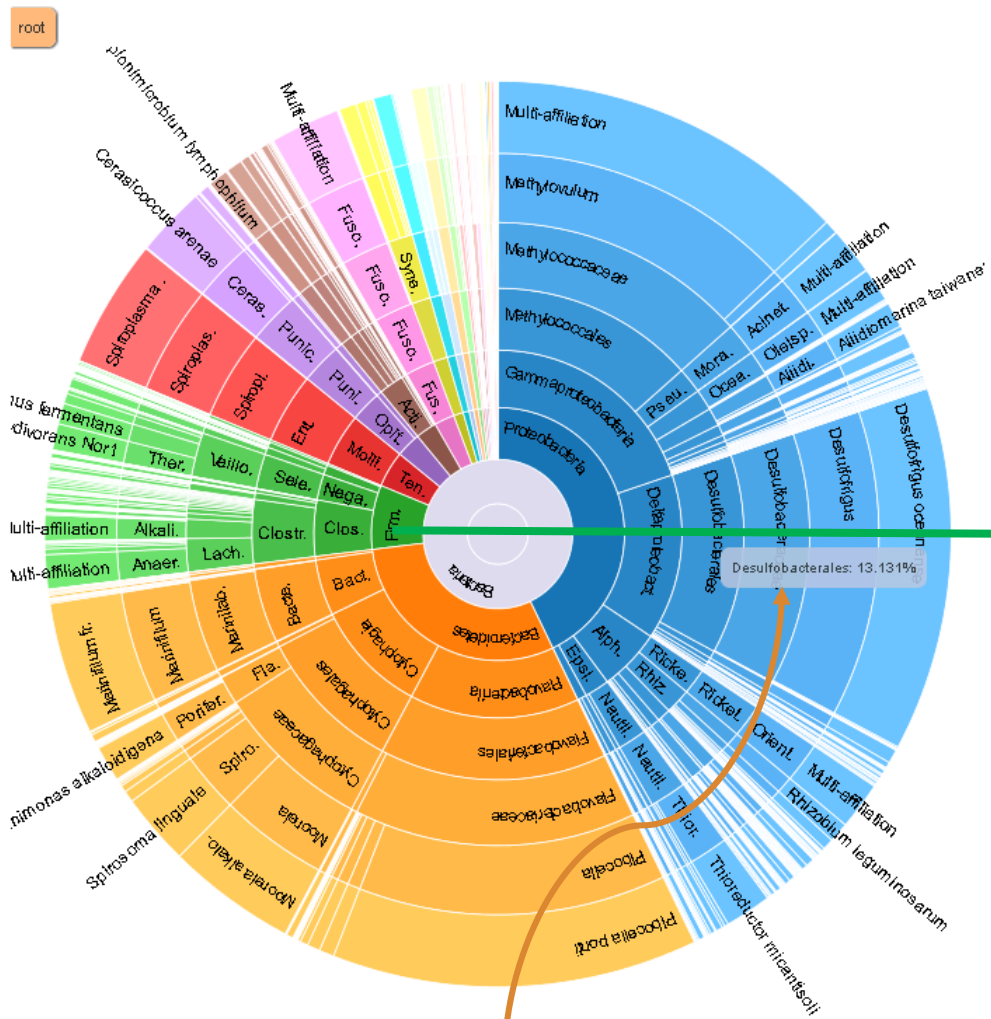
Samples size ~85 000
sequences

Rarefaction



The curve slows to
rise with ~50 000
sequences

With 60 000
sequences, we catch
almost all genus of
bacteria



Zoom in on
firmicutes

Detail on selected:

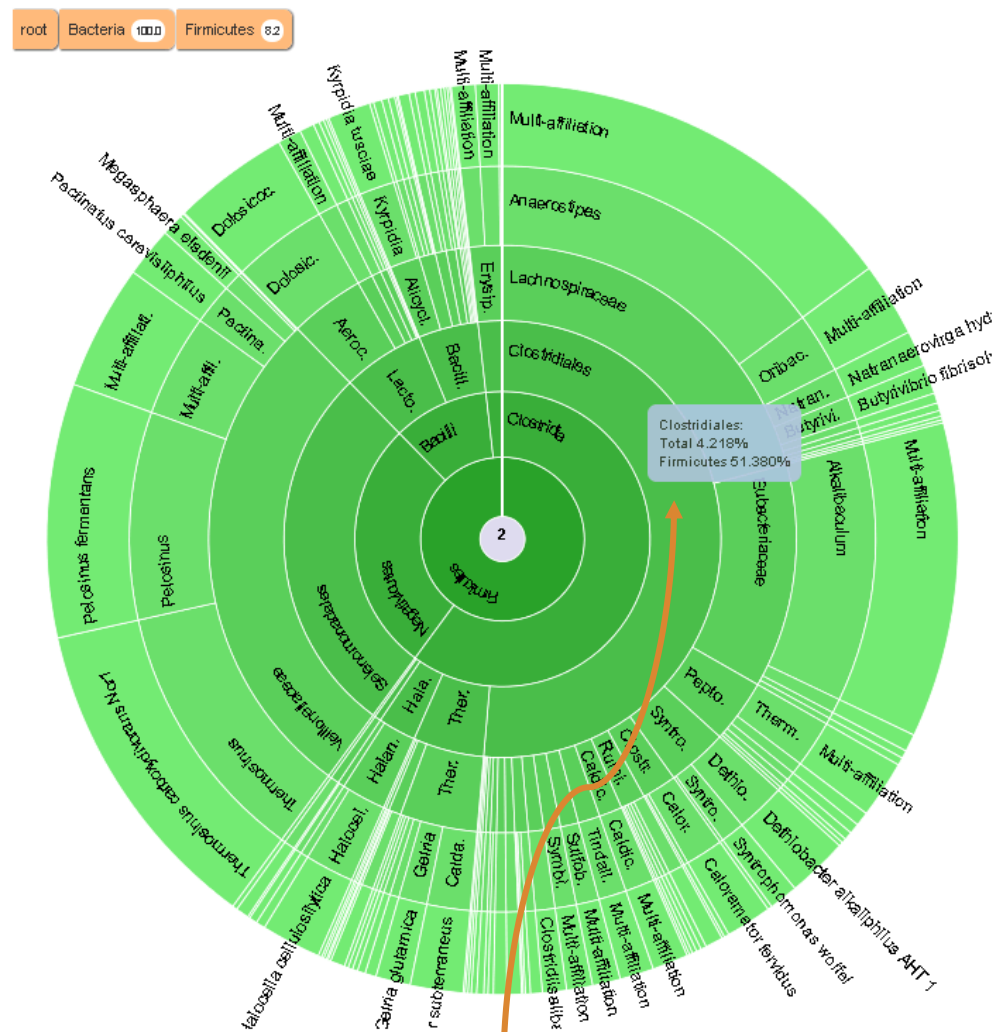
Name	Size	Global %	Parent %
root	246197		
Bacteria	246197	100.000	100.000
Proteobacteria	105524	42.862	42.862
Deltaproteobacteria	35987	14.617	34.103
Desulfobacterales	32328	13.131	89.832

Desulfobacterales nb children: 2

Font size: 15

Colors start depth: 2

Close



Detail on selected:

Name	Size	Global %	Parent %
root	246197		
Bacteria	246197	100.000	100.000
Firmicutes	20212	8.210	8.210
Clostridia	12142	4.932	60.073
Clostridiales	10385	4.218	85.530

Clostridiales nb children: 20

Font size: 15

Colors start depth: 2

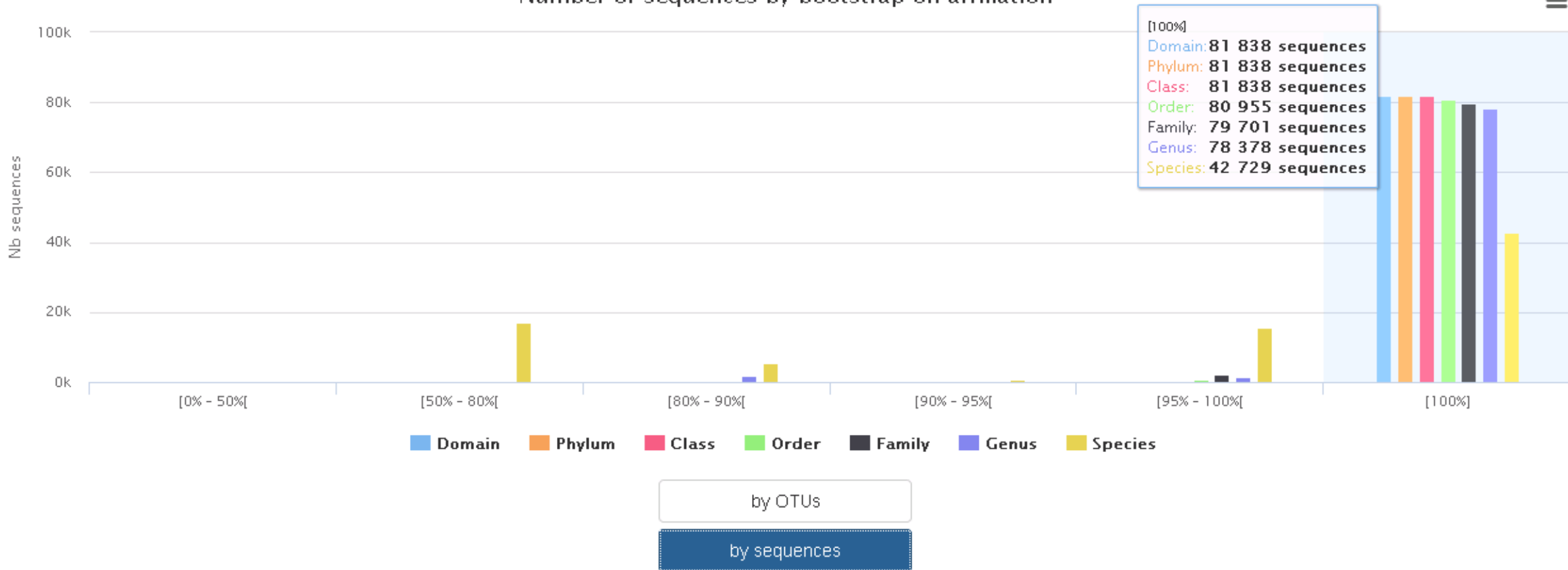
Close

Escape
RDP

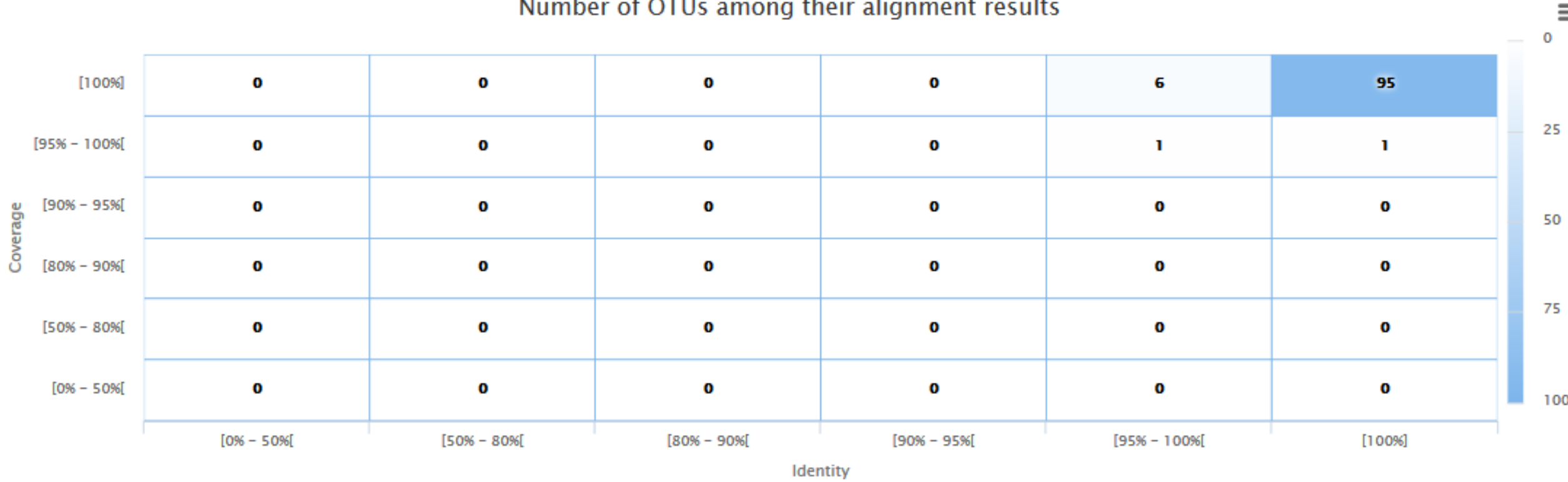
Taxonomy distribution

Bootstrap distribution

Number of sequences by bootstrap on affiliation

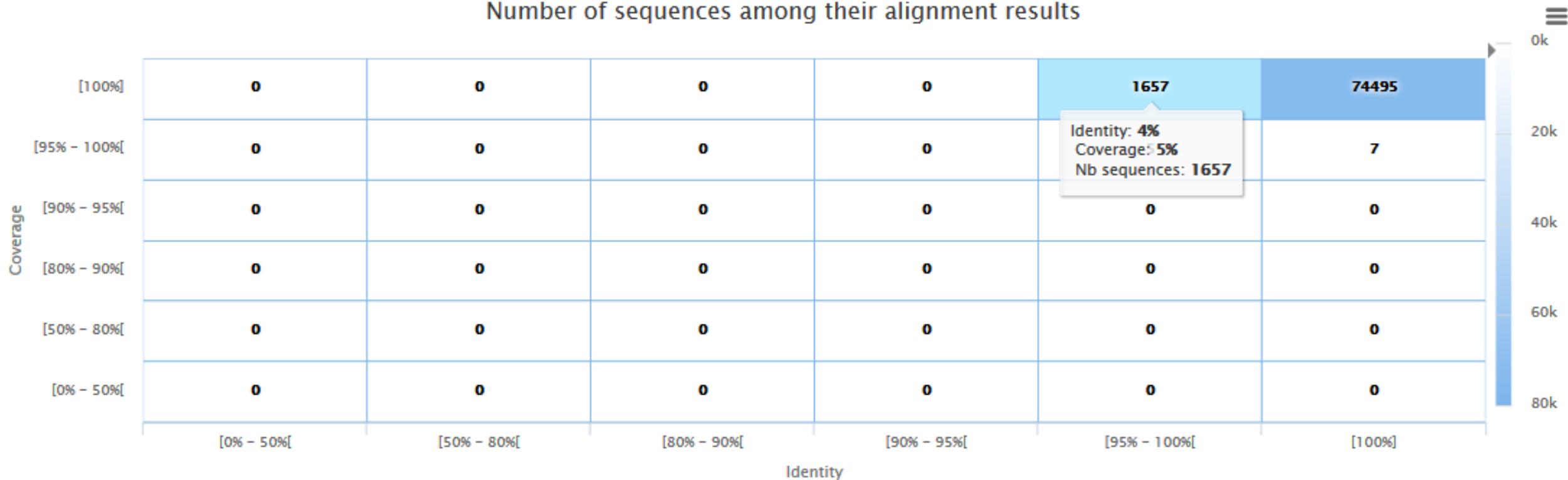


Number of OTUs among their alignment results



by OTUs
by sequences

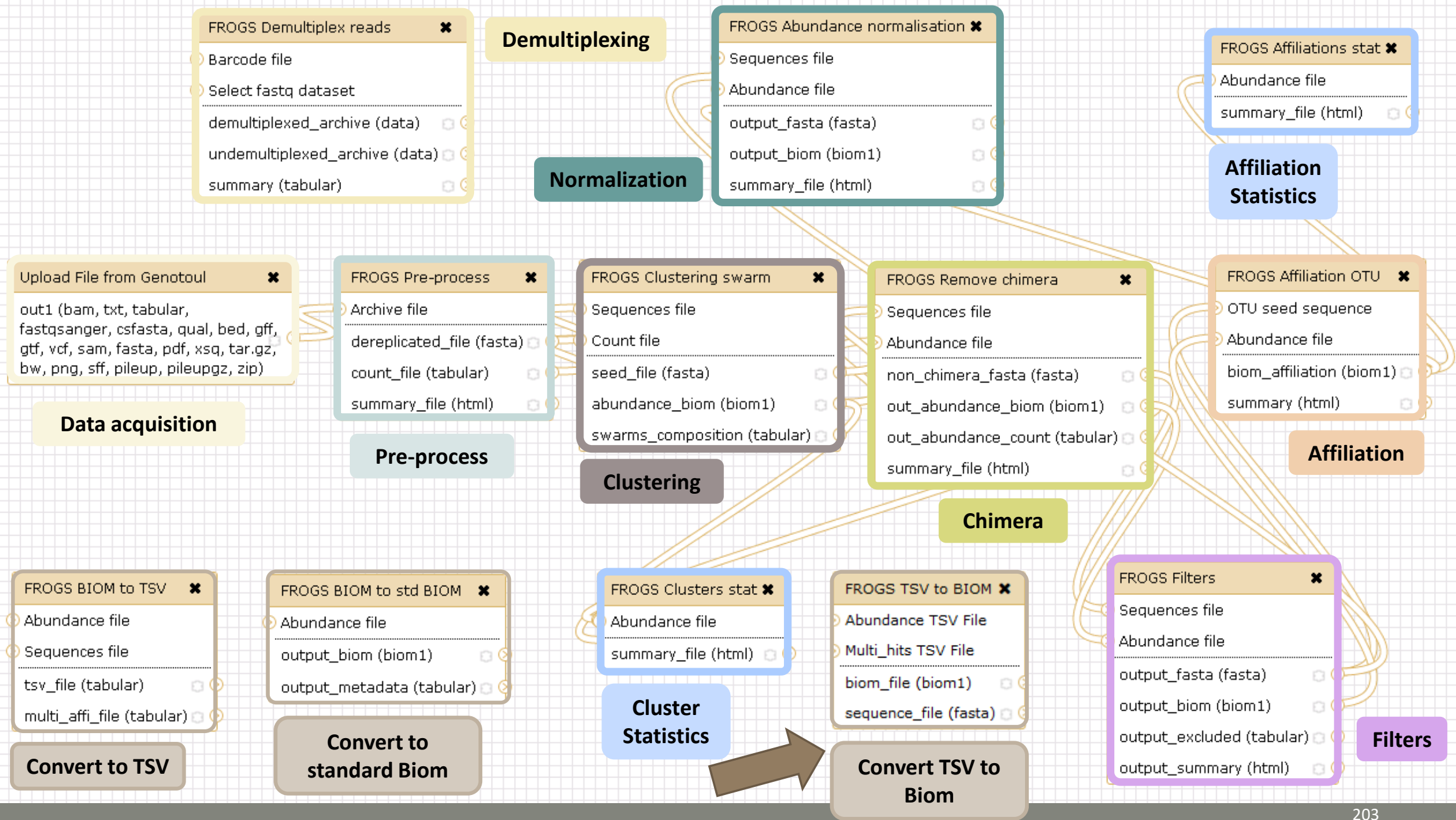
Number of sequences among their alignment results



by OTUs

by sequences

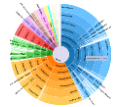
TSV to BIOM



TSV to BIOM

After modifying your abundance TSV file you can again:

- generate rarefaction curve
- sunburst



Careful :

- do not modify column name
- do not remove column
- take care to choose a taxonomy available in your multi_hit TSV file
- if deleting line from multi_hit, take care to not remove a complete cluster without removing all "multi tags" in you abundance TSV file.
- if you want to rename a taxon level (ex : genus "Ruminiclostridium 5;" to genus "Ruminiclostridium;"), do not forget to modify also your multi_hit TSV file.

TSV to BIOM

FROGS TSV_to_BIOM Converts a TSV file in a BIOM file. (Galaxy Version 2.0.0) Options

Abundance TSV File

21: FROGS BIOM to TSV: abundance.tsv

Your FROGS abundance TSV file. Take care to keep original column names.

Multi_hits TSV File

25: multihit_renamed.txt

TSV file describing multi_hit blast results.

Extract seeds in FASTA file

If there is a 'seed_sequence' column in your TSV table, you can extract seed sequences in a separated FASTA file.

Your Turn! – 7

PLAY WITH TSV_TO_BIOM

Exercise 7

→ objectives : Play with multi-affiliation and TSV_to_BIOM

1. Observe in Multi_hit.tsv and abundance.tsv cluster_8 annotation

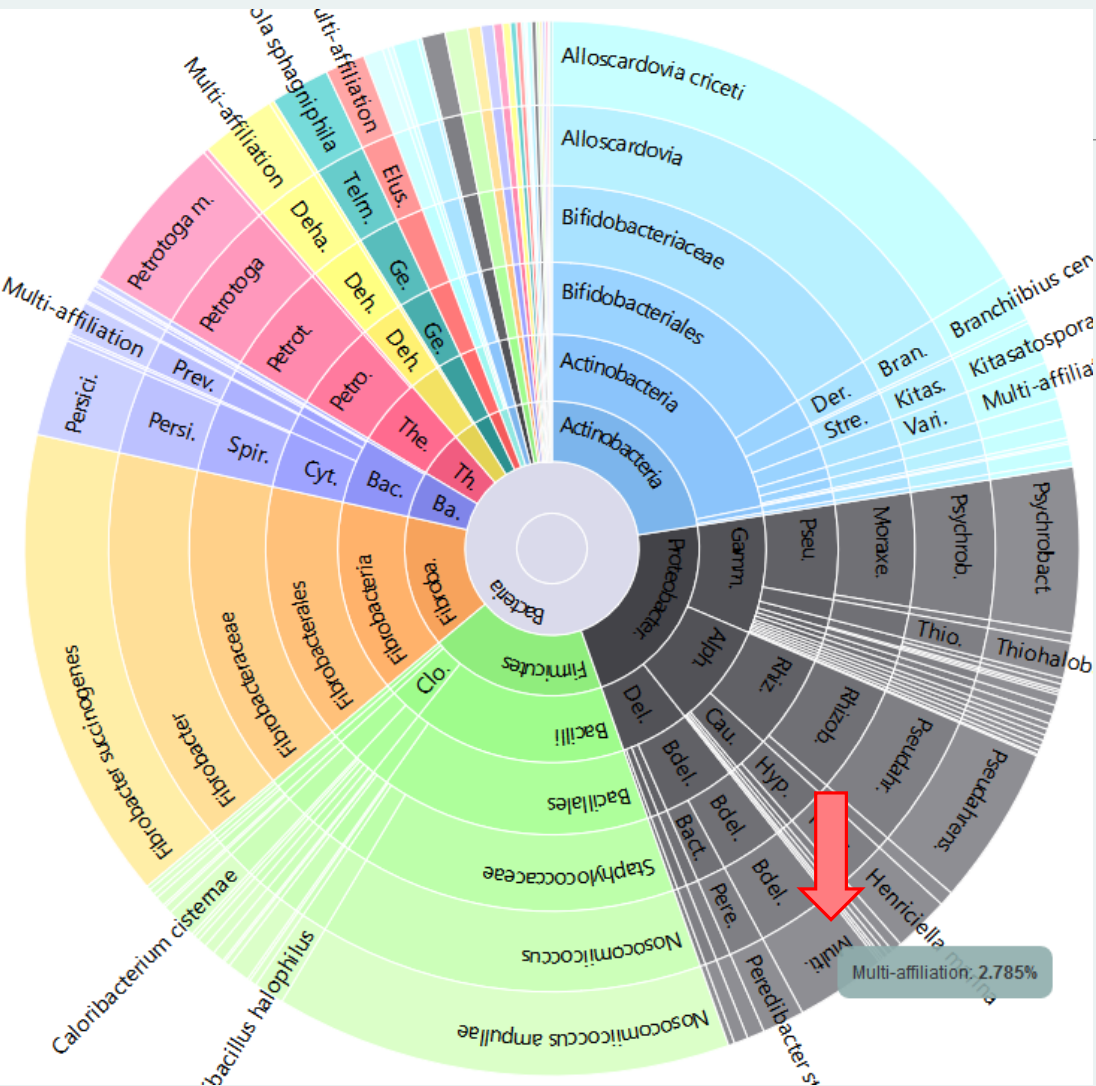
#blast_taxonomy	blast_subject	observation_name	observation_sum
Bacteria;Actinobacteria;Actinobacteria;Bifidobacteriales;Bifidobacteriaceae;Metascardovia;Multi-affiliation	multi-subject	Cluster_1	13337
Bacteria;Fibrobacteres;Fibrobacteria;Fibrobacterales;Fibrobacteraceae;Fibrobacter;Fibrobacter succinogenes	AJ496032.1.1410	Cluster_2	11830
Bacteria;Firmicutes;Bacilli;Bacillales;Staphylococcaceae;Nosocomiicoccus;Nosocomiicoccus ampullae	EU240886.1.1502	Cluster_3	11405
Bacteria;Proteobacteria;Gammaproteobacteria;Pseudomonadales;Moraxellaceae;Psychrobacter;Psychrobacter immobilis	U39399.1.1477	Cluster_4	4125
Bacteria;Thermotogae;Thermotogae;Thermotogales;Thermotogaceae;Petrotoga;Petrotoga miotherma	FR733705.1.1499	Cluster_5	4034
Bacteria;Proteobacteria;Alphaproteobacteria;Rhizobiales;Phyllobacteriaceae;Pseudahrensia;Pseudahrensia aquimaris	GU575117.1.1441	Cluster_6	3966
Bacteria;Bacteroidetes;Cytophagia;Cytophagales;Cytophagaceae;Persicitalea;Persicitalea jodogahamensis	multi-subject	Cluster_7	2433
Bacteria;Proteobacteria;Deltaproteobacteria;Bdellovibrionales;Bdellovibrionaceae;Bdellovibrio;Multi-affiliation	multi-subject	Cluster_8	2268

Cluster_8	Bacteria;Proteobacteria;Deltaproteobacteria;Bdellovibrionales;Bdellovibrionaceae;Bdellovibrio;Bdellovibrio bacteriovorus	CP007656.1036900.1038415
Cluster_8	Bacteria;Proteobacteria;Deltaproteobacteria;Bdellovibrionales;Bdellovibrionaceae;Bdellovibrio;Bdellovibrio bacteriovorus str. Tiberius	CP002930.1837665.1839157
Cluster_8	Bacteria;Proteobacteria;Deltaproteobacteria;Bdellovibrionales;Bdellovibrionaceae;Bdellovibrio;Bdellovibrio bacteriovorus str. Tiberius	CP002930.842397.843889
Cluster_8	Bacteria;Proteobacteria;Deltaproteobacteria;Bdellovibrionales;Bdellovibrionaceae;Bdellovibrio;Bdellovibrio bacteriovorus	AJ292760.1.1334
Cluster_8	Bacteria;Proteobacteria;Deltaproteobacteria;Bdellovibrionales;Bdellovibrionaceae;Bdellovibrio;Bdellovibrio bacteriovorus	AF084850.1.1436
Cluster_8	Bacteria;Proteobacteria;Deltaproteobacteria;Bdellovibrionales;Bdellovibrionaceae;Bdellovibrio;Bdellovibrio bacteriovorus HD100	BX842648.123565.125058
Cluster_8	Bacteria;Proteobacteria;Deltaproteobacteria;Bdellovibrionales;Bdellovibrionaceae;Bdellovibrio;Bdellovibrio bacteriovorus HD100	BX842650.295616.297109



Bdellovibrio bacteriovorus

2. Observe le diversity diagramm



Exercise 7

3. How to change affiliation of cluster 8 ????

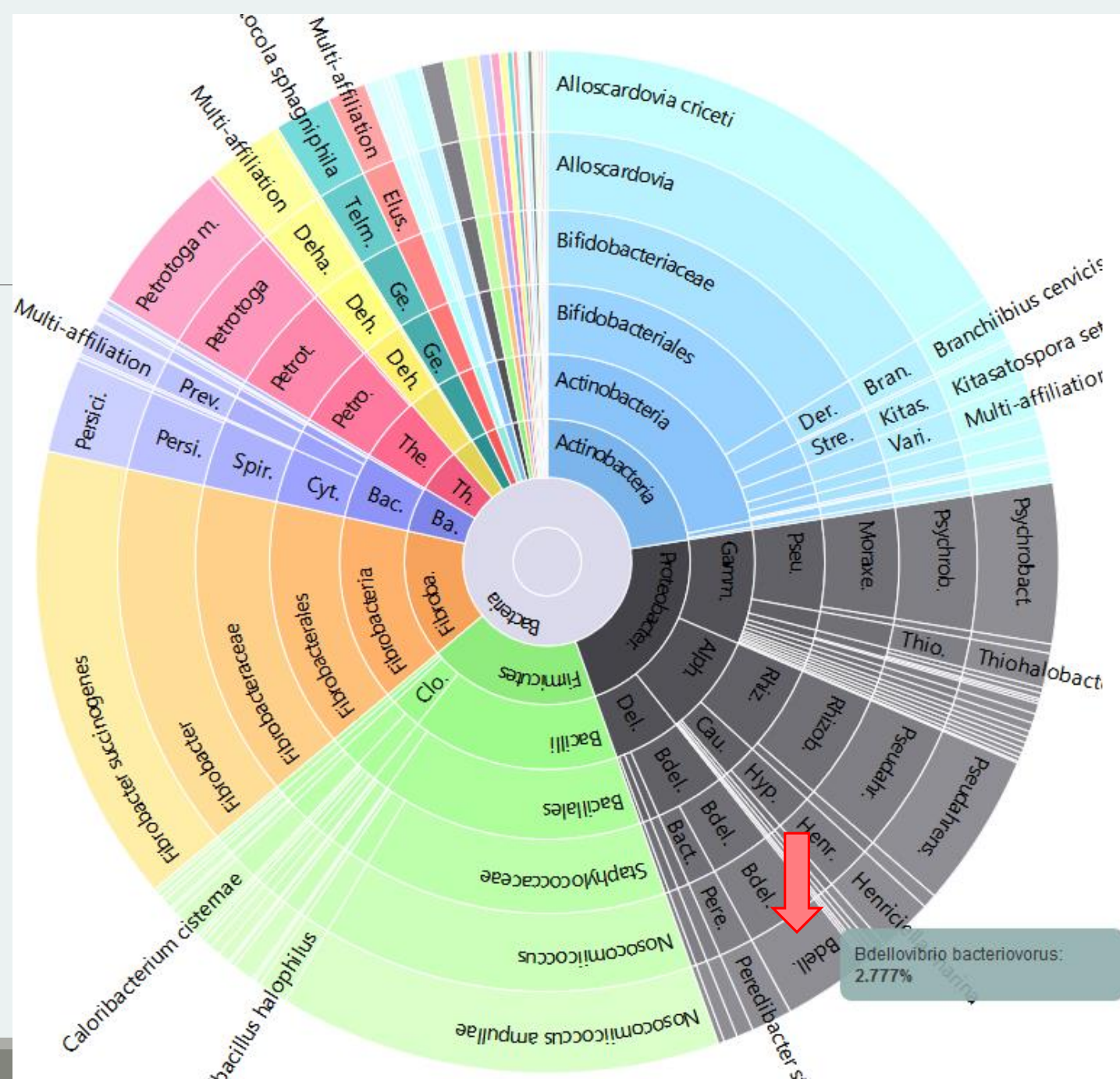
Exercise 7

4. Modify multi_hit.tsv under excel for example and keep only :

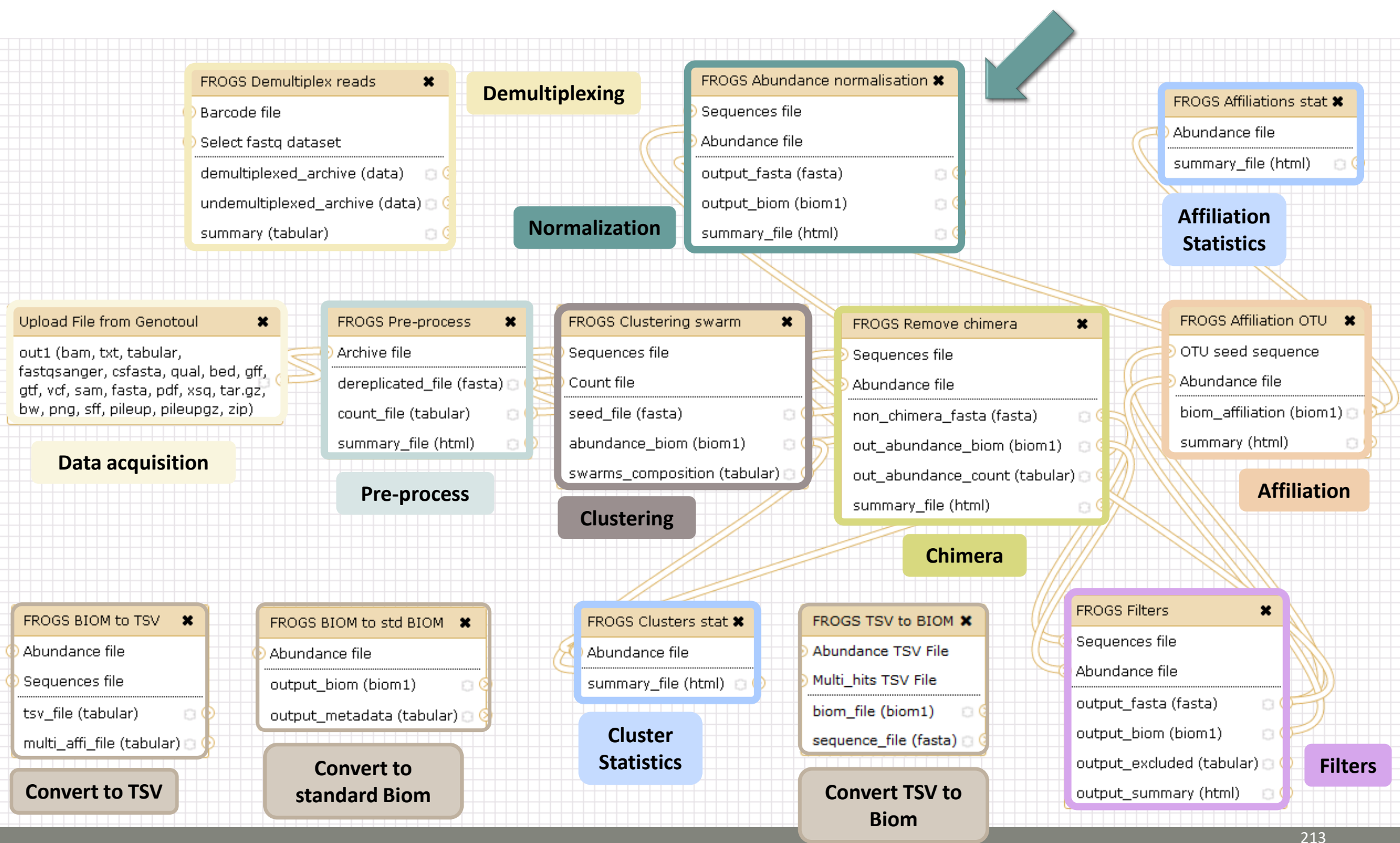
Cluster_8 Bacteria;Proteobacteria;Deltaproteobacteria;Bdellovibrionales;Bdellovibrionaceae;Bdellovibrio;Bdellovibrio bacteriovorus CP007656.1036900.1038415

5. Save in multihit_cluster8_modified.tsv
6. Upload the new multihit file.
7. Create a new biom with a TSV_to_BIOM tool
8. Launch again the affiliation_stat tool on this new biom
9. Observe the diversity diagram

Exercise 7



Normalization



Normalization

Conserve a predefined number of sequence per sample:

- update Biom abundance file
- update seed fasta file

May be used when :

- Low sequencing sample
- Required for some statistical methods to compare the samples in pairs

Your Turn! – 8

LAUNCH NORMALIZATION TOOL

Exercise 8

Launch Normalization Tool

1. What is the smallest sequenced samples ?
2. Normalize your data from Affiliation based on this number of sequence
3. Explore the report HTML result.
4. Try other threshold and explore the report HTML result
What do you remark ?

FROGS Abundance normalisation (Galaxy Version r3.0-8.0) Options

Sequence file

16: FROGS Filters: sequences.fasta

Sequence file to normalize (format: fasta).

Abundance file

21: FROGS Affiliation OTU: affiliation.biom

Abundance file to normalize (format: BIOM).

Number of reads

9029

The final number of reads per sample.

FROGS Abundance normalisation (Galaxy Version 1.1.1) Options

Sequences file

17: FROGS Filters: sequences.fasta

Sequences file to normalize (format: fasta).

Abundance file

22: FROGS Affiliation OTU: affiliation.biom

Abundances file to normalize (format: BIOM).

Number of reads

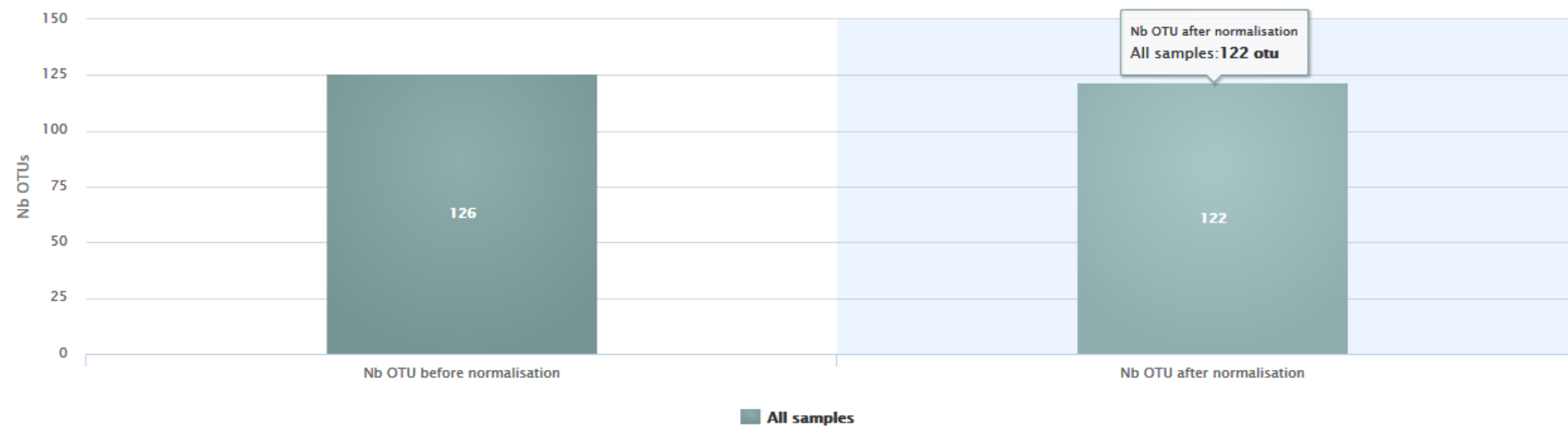
2000

The final number of reads per sample.

Execute

Or, this number can be chosen according to the rarefaction curve. For example, we can choose the smallest number of sequences that still retain all the genus.

Composition summary



Show entries

Search:

Sample	Nb OTU before normalisation	Nb OTU after normalisation
100_10000seq_sampleA1	126	122
100_10000seq_sampleA2	126	122
100_10000seq_sampleA3	126	122
100_10000seq_sampleB1	126	122
100_10000seq_sampleB2	126	122
100_10000seq_sampleB3	126	122
100_10000seq_sampleC1	126	122
100_10000seq_sampleC2	126	122
100_10000seq_sampleC3	126	122

Filters on affiliations

Do not forget, with filter tool we can filter the data based on their affiliation

FROGS Filters Filters OTUs on several criteria. (Galaxy Version 1.2.0) Options

Sequences file
9: FROGS Remove chimera: non_chimera.fasta
The sequence file to filter (format: fasta).

Abundance file
10: FROGS Remove chimera: non_chimera_abundance.biom
The abundance file to filter (format: BIOM).

***** THE FILTERS ON OTUS IN SAMPLES, OTUS SIZE and SEQUENCE PERCENTAGE**

Apply filters
If you want to filter OTUs on their abundance and occurrence.

Minimum number of samples
Fill the field only if you want this treatment. Keep OTU present in at least this number of samples.

Minimum proportion/number of sequences to keep OTU
Fill the field only if you want this treatment. Use decimal notation for proportion (example: 0.01 for keep OTU with at least 1% of all sequences) ; Use integer notation for number of sequence (example: 2 for keep OTU with at least 2 sequences, so remove single singleton).

N biggest OTU
Fill the fields only if you want this treatment. Keep the N biggest OTU.

***** THE FILTERS ON RDP**

Apply filters
If you want to filter OTUs on their taxonomic affiliation produced by RDP.

Rank with the bootstrap filter
Nothing selected

Minimum bootstrap % (between 0 and 1)

***** THE FILTERS ON BLAST**

Apply filters
If you want to filter OTUs on their taxonomic affiliation produced by Blast.

Maximum e-value (between 0 and 1)
Fill the field only if you want this treatment

Minimum identity % (between 0 and 1)
Fill the field only if you want this treatment

Minimum coverage % (between 0 and 1)
Fill the field only if you want this treatment

Minimum alignment length
Fill the field only if you want this treatment

***** THE FILTERS ON CONTAMINATIONS**

Apply filters
If you want to filter OTUs on classical contaminations.

Cotaminant databank
phix
The phix databank (the phix is a control added in Illumina sequencing technologies).

Execute

Abundance filters

RDP affiliation filters

BLAST affiliation filters

Contamination filter

Exercise 9

1. Apply filters to keep only data with perfect alignment.
2. How many clusters have you keep ?

Sequences file

17: FROGS Filters: sequences.fasta

The sequence file to filter (format: fasta).

Abundance file

22: FROGS Affiliation OTU: affiliation.biom

The abundance file to filter (format: BIOM).

***** THE FILTERS ON OTUS IN SAMPLES, OTUS SIZE and SEQUENCE PERCENTAGE**

No filters

If you want to filter OTUs on their abundance and occurrence.

***** THE FILTERS ON RDP**

No filters

If you want to filter OTUs on their taxonomic affiliation produced by RDP.

***** THE FILTERS ON BLAST**

Apply filters

If you want to filter OTUs on their taxonomic affiliation produced by Blast.

Maximum e-value (between 0 and 1)

Fill the field only if you want this treatment

Minimum identity % (between 0 and 1)

1

Fill the field only if you want this treatment

Minimum coverage % (between 0 and 1)

1

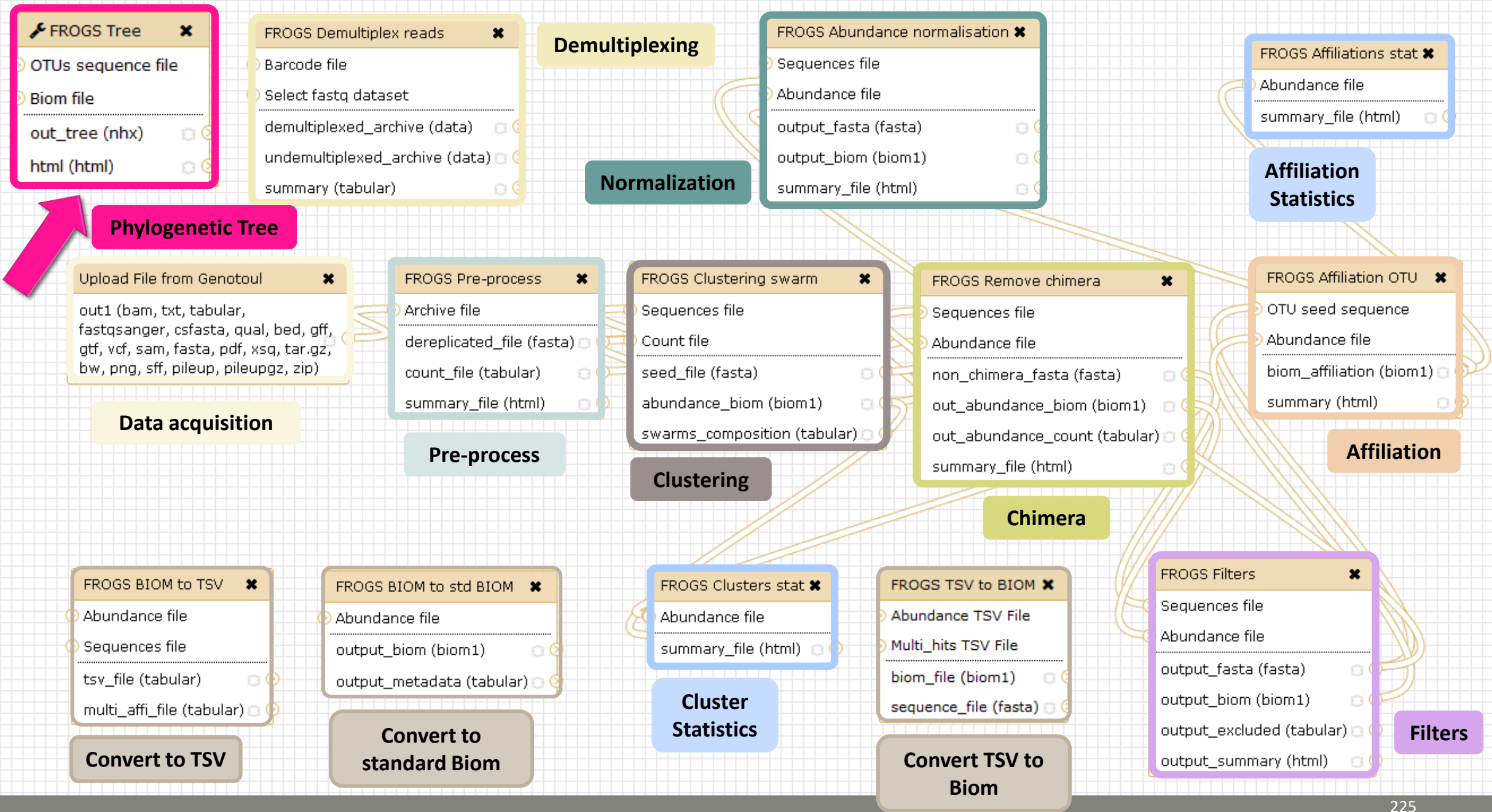
Fill the field only if you want this treatment

Minimum alignment length

Fill the field only if you want this treatment

FROGS Tree

CREATE A PHYLOGENETICS TREE OF OTUS



2 choices to do your phylogenetics tree

FROGS Tree Reconstruction of phylogenetic tree (Galaxy Version 1.0.0) Options

OTUs sequence file
12: FROGS Filters: sequences.fasta
OTUs sequence file (format: fasta). Warning: FROGS Tree does not work on more than 10000 sequences!

Do you have the template alignment file ?
Yes **No**
If yes, precise the template multi-alignment file.

Biom file
16: FROGS Affiliation OTU: affiliation.biom
The abundance table of OTUs (format: biom).

Execute

FROGS Tree Reconstruction of phylogenetic tree (Galaxy Version 1.0.0) Options

OTUs sequence file
12: FROGS Filters: sequences.fasta
OTUs sequence file (format: fasta). Warning: FROGS Tree does not work on more than 10000 sequences!

Do you have the template alignment file ?
Yes No
If yes, precise the template multi-alignment file.

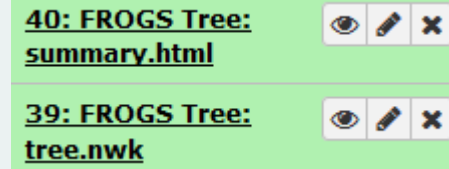
Template alignment file
22: otus_pynast.fasta
Template multi-alignment file (format: fasta).

Biom file
16: FROGS Affiliation OTU: affiliation.biom
The abundance table of OTUs (format: biom).

Execute

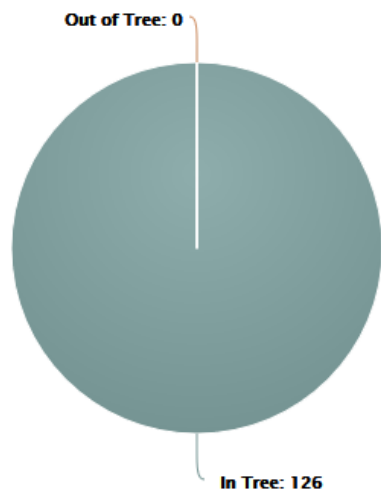
Exercise 9

1. Create a tree with the filtered OTUs without template
2. Explore the HTML file
3. Look tree.nwk

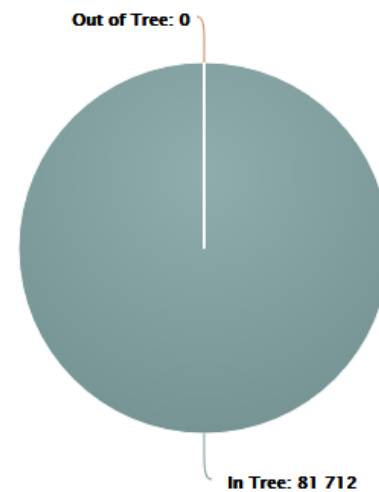


Summary

OTUs

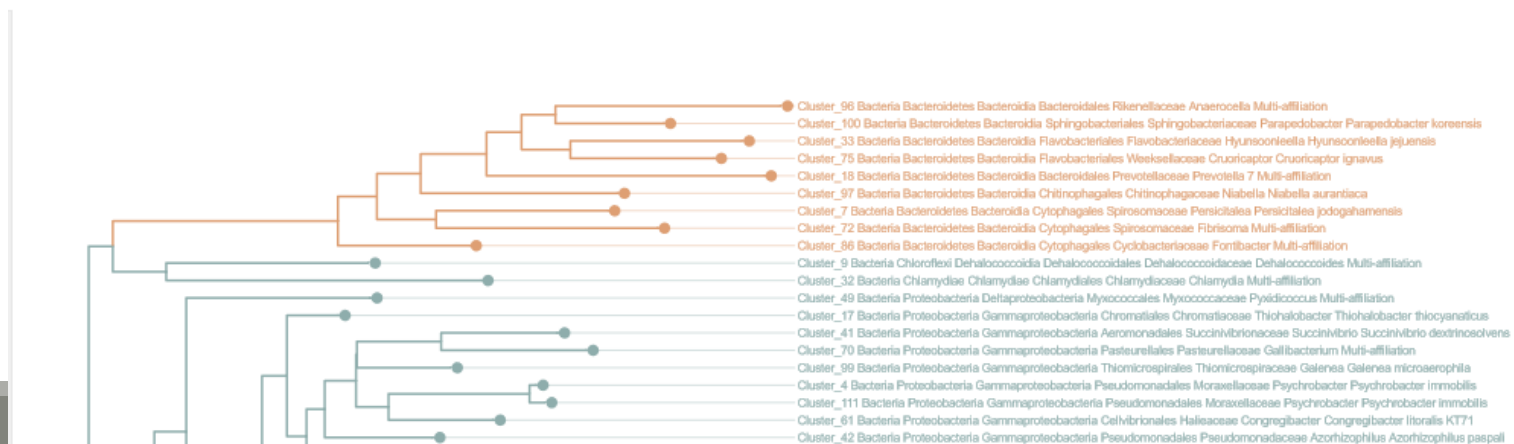


Abundance



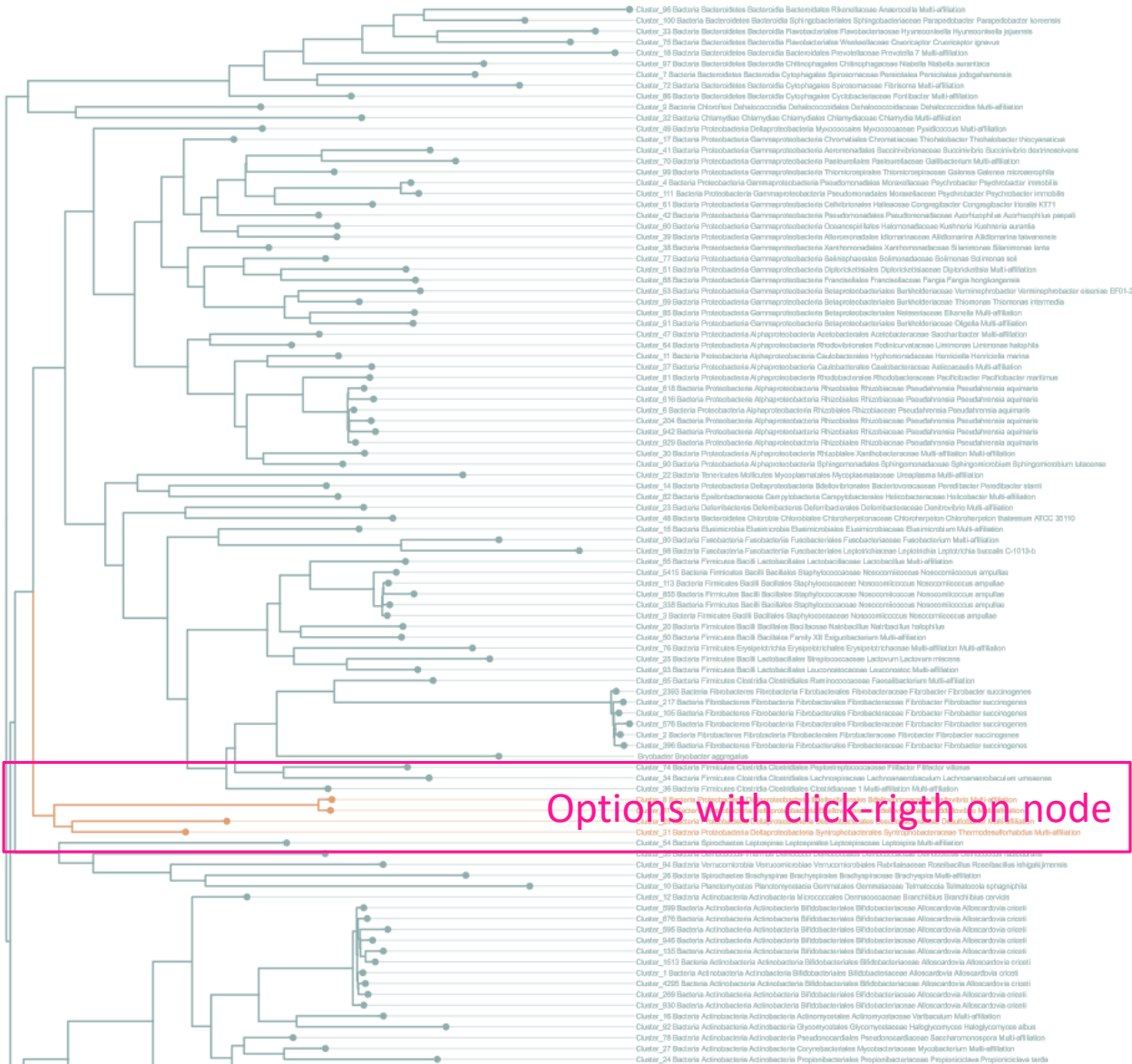
Tree View

Enabling zoom:



Tree View

Enabling zoom:



Options with click-righ on node

Tree.nwk:

((Cluster_8 Bacteria Proteobacteria Deltaproteobacteria Bdellovibrionales Bdellovibrionaceae Bdellovibrio Multi-affiliation:0.00879,Cluster_117 Bacteria Proteobacteria Deltaproteobacteria Bdellovibrionales Bdellovibrionaceae Bdellovibrio Multi-affiliation:0.00744):0.25827,(Cluster_28 Bacteria Proteobacteria Deltaproteobacteria Desulfobacteriales Desulfobacteraceae Desulfobacter Multi-affiliation:0.14675,Cluster_31 Bacteria Proteobacteria Deltaproteobacteria Syntrophobacteriales Syntrophobacteraceae Thermodesulforhabdus Multi-affiliation:0.10644):0.01759):0.02059;

How works FROGS TREE ?

Pynast needs alignment template to go fast

But if your species is not similar at 75% with a sequence in the template, your species will be not in the tree !

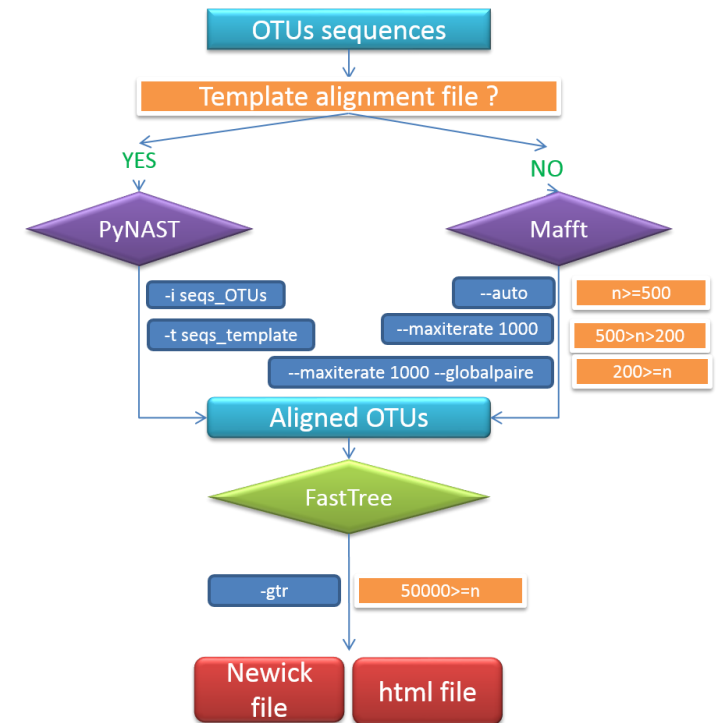
To find templates:

Based on 16S GreenGenes databank

https://github.com/biocore/qiime-default-reference/blob/master/qiime_default_reference/gg_13_8_otus/rep_set_aligned/85_otus.pynast.fasta.gz

Based on 16S SILVA databank

https://www.arb-silva.de/fileadmin/silva_databases/qiime/Silva_128_release.tgz



Tool descriptions

Example of Preprocess tool HELP



i What it does

FROGS Pre-process filters and dereplicates amplicons for use in diversity analysis.

i Inputs/Outputs

Inputs

Sample files added one after another or provide in an archive file (tar.gz).

ILLUMINA inputs

Usage: For samples sequenced in paired-end. The amplicon length must be inferior to the length of the R1 plus R2 length. R1 and R2 are merged by the common region.

Files: One R1 and R2 by sample (format [FASTQ](#))

Example: splA_R1.fastq.gz, splA_R2.fastq.gz, splB_R1.fastq.gz, splB_R2.fastq.gz

OR

Usage: For samples sequenced in single-ends or when R1 and R2 reads are already merged.

Files: One sequence file by sample (format [FASTQ](#)).

Example: splA.fastq.gz, splB.fastq.gz

454 inputs

Files: One sequence file by sample (format [FASTQ](#))

Example: splA.fastq.gz, splB.fastq.gz

Remark: In an archive if you use R1 and R2 files they names must end with `_R1` and `_R2`.

Outputs

Sequence file (dereplicated.fasta):

Only one file with all samples sequences (format [FASTA](#)). These sequences are dereplicated: strictly identical sequence are represent

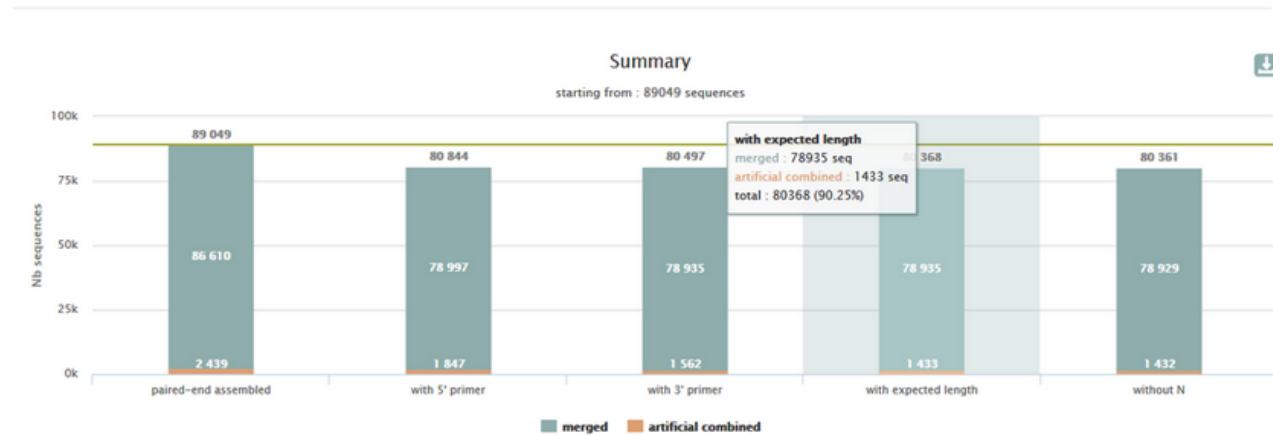
Count file (count.tsv):

This file contains the count of all unique sequences in each sample (format [TSV](#)).

Summary file (report.html):

This file reports the number of remaining sequences after each filter (format [HTML](#)).

Preprocess summary



Details on merged sequences

Show entries Search: [CSV](#)

<input type="checkbox"/>	Samples	% kept	paired-end assembled	with 5' primer	with 3' primer	with expected length	without N
<input checked="" type="checkbox"/>	echantillon1-1	84.93	31,836	27,059	27,040	27,040	27,039
<input type="checkbox"/>	echantillon1-2	94.73	54,774	51,938	51,895	51,895	51,890

With selection: [Display amplicon lengths](#) [Display preprocessed amplicon lengths](#)

Showing 1 to 2 of 2 entries

[Previous](#) [Next](#)

i How it works

Steps	Illumina	454
1	For un-merged data: merges R1 and R2 with a maximum of M% mismatch in the overlaped region(VSEARCH or FLASH or optionnaly PEAR). Resulting un-merged reads may optionnaly be artificially combined by adding 100 N between the reads	/
2	If sequencing protocol is the illumina standard protocol : Removes sequences where the two primers are not present and then remove primers in the remaining sequence (cutadapt). The primer search accepts 10% of differences	Removes sequences where the two primers are not present, removes primers sequence and reverse complement the sequences on strand - (cutadapt). The primer search accepts 10% of differences
3	Filters sequences with ambiguous nucleotides and for merged sequences filters on their length which must be range between 'Minimum amplicon size - primer length' and 'Maximum amplicon size - primer length'	the tool removes sequences with at least one homopolymer with more than seven nucleotides and with a distance of less than or equal to 10 nucleo-tides between two poor quality positions, i.e. with a Phred quality score lesser than 10
4	Dereplicates sequences	Dereplicates sequences

i Advices/details on parameters

Primers parameters

The primers must be provided in 5' to 3' orientation.

Example:

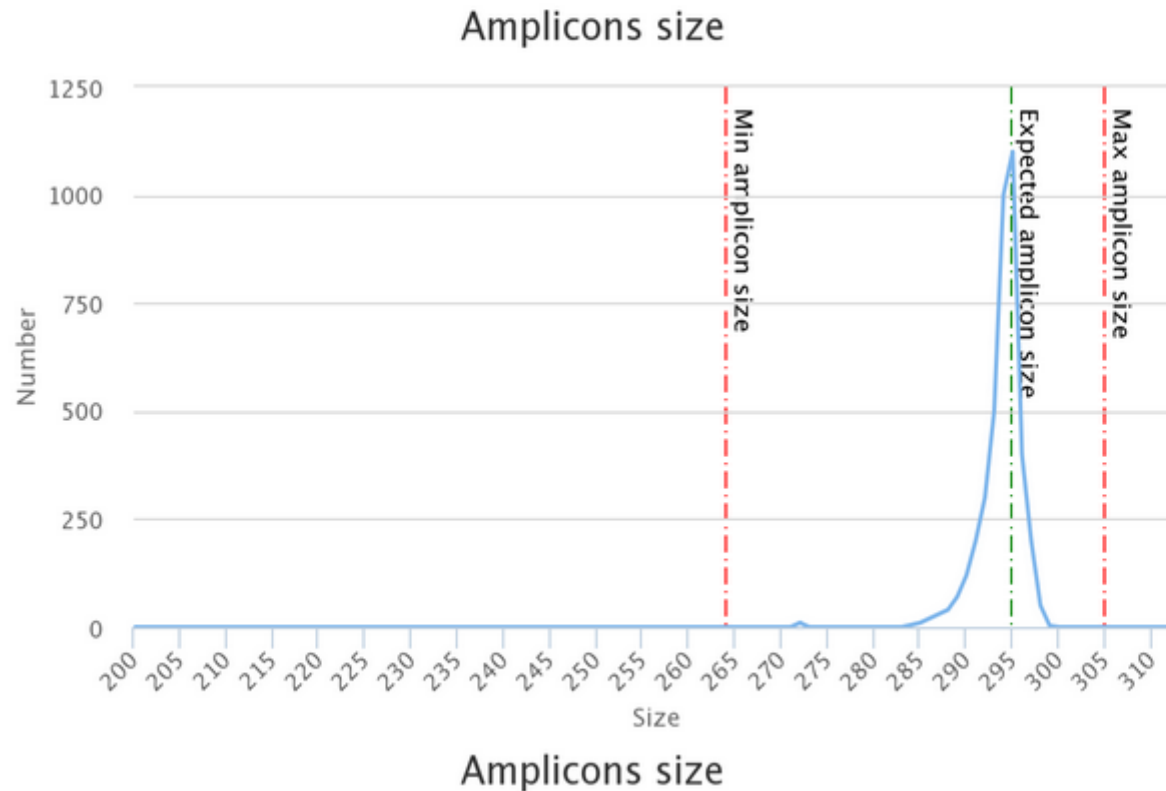
5' **ATGCC** GTCGTCGTAAAATGC **ATTCAG** 3'

Value for parameter 5' primer: ATGCC

Value for parameter 3' primer: ATTCAG

Amplicons sizes parameters

The two following images shown two examples of perfect values for sizes parameters.



i Advices/details on parameters

What is the difference between overlapped sequences and combined sequences?

Case of a sequencing of overlapping sequences: case of 16S V3-V4 amplicon MiSeq sequencing

Imagine a real amplicon sequence of 400bp



Imagine a MiSeq paired sequencing of 2x250bp



Reconstructing amplicon sequence is possible thanks to the overlap region



Merged sequence length : 400bp, with 100bp overlap

Case of a sequencing of non-overlapping sequences: case of ITS1 amplicon MiSeq sequencing

Imagine a real amplicon sequence of 700bp



Imagine a MiSeq paired sequencing of 2x250bp



Reconstructing amplicon sequence is not possible with overlap, an arbitrary sequence of 100Ns is added. It is named « FROGS combined »



Combined sequence length : 600bp, with 100 Ns

⚠ "FROGS combined" warning points

Reads pair are not merged because:

- the real amplicon length is greater than the number of base sequences (500 bp for MiSeq 2x250bp)
- the overlapped region is smaller than 10 (fixed parameter in FROGS).

Thus, "FROGS combined" sequences are artificial and present particular features especially on size. Imagine a MiSeq sequencing of 2x250 sequences length will be 600 bp.

Case 1: real amplicon > 601 bp → "FROGS combined" length is smaller than the reality

Contact

Contacts: frogs@inra.fr

Repository: <https://github.com/geraldinepascal/FROGS> website: <http://frogs.toulouse.inra.fr/>

Please cite the **FROGS article**: *Escudie F., et al. Bioinformatics, 2018. FROGS: Find, Rapidly, OTUs with Galaxy Solution.*




Download your data

In order to share resources as well as possible, files that have not been accessed for more than 120 days are regularly purged. The backup of data generated using of Galaxy is your responsibility.






You have the opportunity:

1/ Save your datasets one by one using the "floppy disk" icon.



55: FROGS Affiliation   

OTU:
excluded_data_report.html
11.4 KB
format: html, database: ?
Application Software:
affiliation_OTU.py (version: 0.4.0)
Command: /usr/local/bioinfo
/src/galaxy-test/galaxy-dist/tools
/FROGS/affiliation_OTU.py
--reference /save/galaxy-
test/bank/FROGS/silva_119-1
/prokaryotes
/silva_119-1_prokaryotes.fasta
--abundance



    

HTML file

2/ Or export each history.

To export a history, from the "History" menu, click on the wheel, then "Export History to File":



History  

HISTORY LISTS

- Saved Histories
- Histories Shared with Me

HISTORY ACTIONS

- Create New
- Copy History
- Share or Publish
- Show Structure
- Extract Workflow
- Delete
- Delete Permanently

DATASET ACTIONS

- Copy Datasets
- Dataset Security
- Resume Paused Jobs
- Collapse Expanded Datasets
- Unhide Hidden Datasets
- Delete Hidden Datasets
- Purge Deleted Datasets

DOWNLOADS

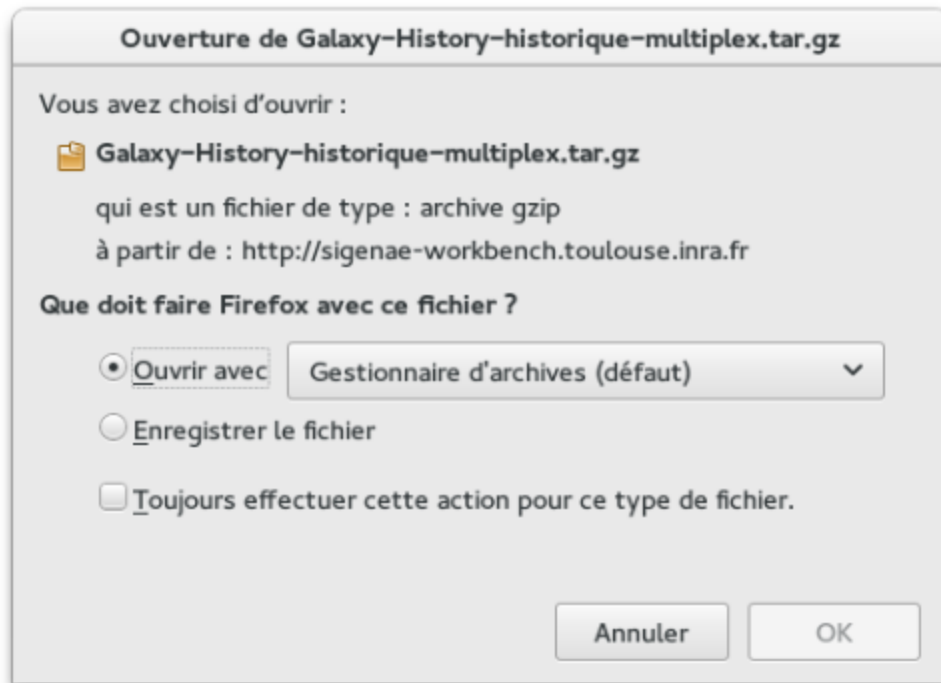
- Export Tool Citations
- Export History to File**

OTHER ACTIONS

- Import from File

To retrieve your history, click on the http link that appears automatically:

It is then possible to record the data :



This directory contains :



1. in the "datasets" directory: Your Galaxy files.
2. in the files "-attrs.txt" : Metadata about your datasets, your jobs and your history.




FROGS BIOM to Standard BIOM

FROGS biom to standard Biom

This step is required to run R

FROGS BIOM to std BIOM Converts a FROGS BIOM in fully compatible BIOM. (Galaxy Version 1.1.0) Options

Abundance file

   22: FROGS Affiliation OTU: affiliation.biom

The FROGS BIOM file to convert (format: BIOM).



43: FROGS BIOM to std BIOM: blast_metadata.tsv   

42: FROGS BIOM to std BIOM: abundance.biom   

Some figures

Some figures - Fast

NB SEQ	TIME with complete pipeline without Filters
50 000	40 min
400 000	4 hrs
3 500 000	2 days
10 000 000	5 days

Speed on real datasets **with filter**

Escape statistics on assessments

9 600 000 sequences of a complete MiSeq run

Preprocess : 9 300 000 sequences

~ 15 min

Swarm clustering : 680 000 clusters

~ 10 hrs

Chimera removal : 556 700 non-chimeric cl.

~ 15 min

Filtering* : 556 200 OTUs

*Filter OTU abundances at 0.005%

PhiX removal

~ 8 min

RDP affiliation

~ 25 min

Blast affiliation

~ 5 min

FROGS : 500 OTUs

~ 11 hours

Simulated datasets, for testing FROGS' Accuracy

- 500 species, covering all bacterial phyla
- Power Law distribution of the species abundances
- Error rate calibrated with real sequencing runs
- 20% chimeras
- 10 samples of 100 000 sequences each (1M sequences)

Simulated dataset : 1M sequences



SWARM : 109 000 clusters

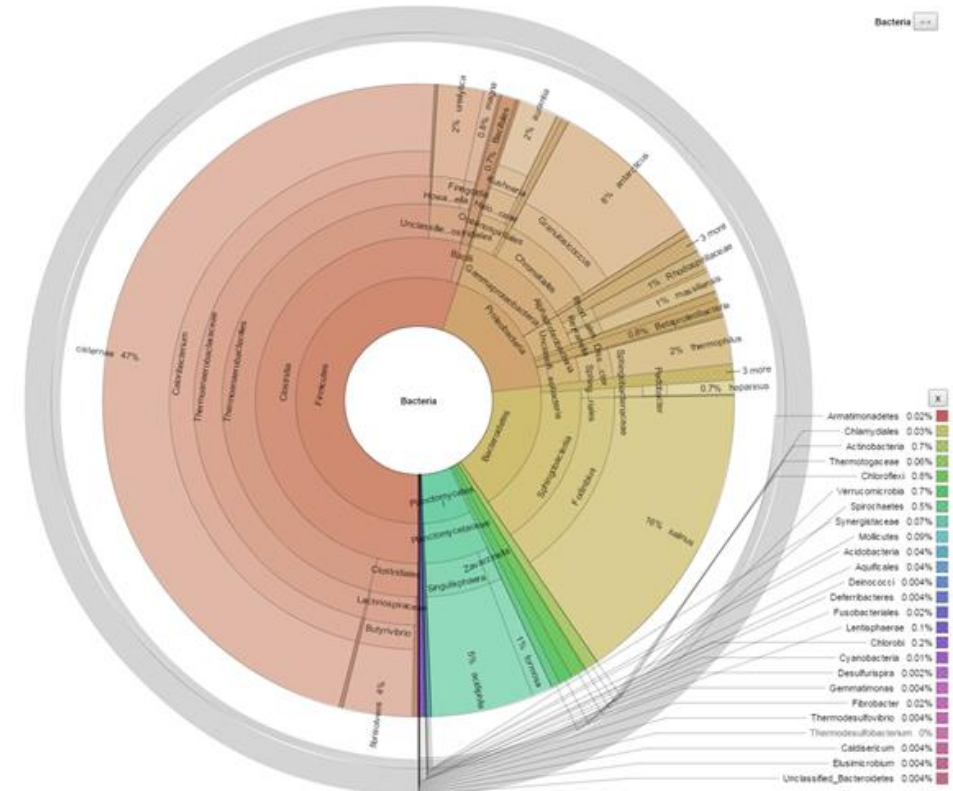


VSEARCH: 21 000 clusters



filters : 0.005%

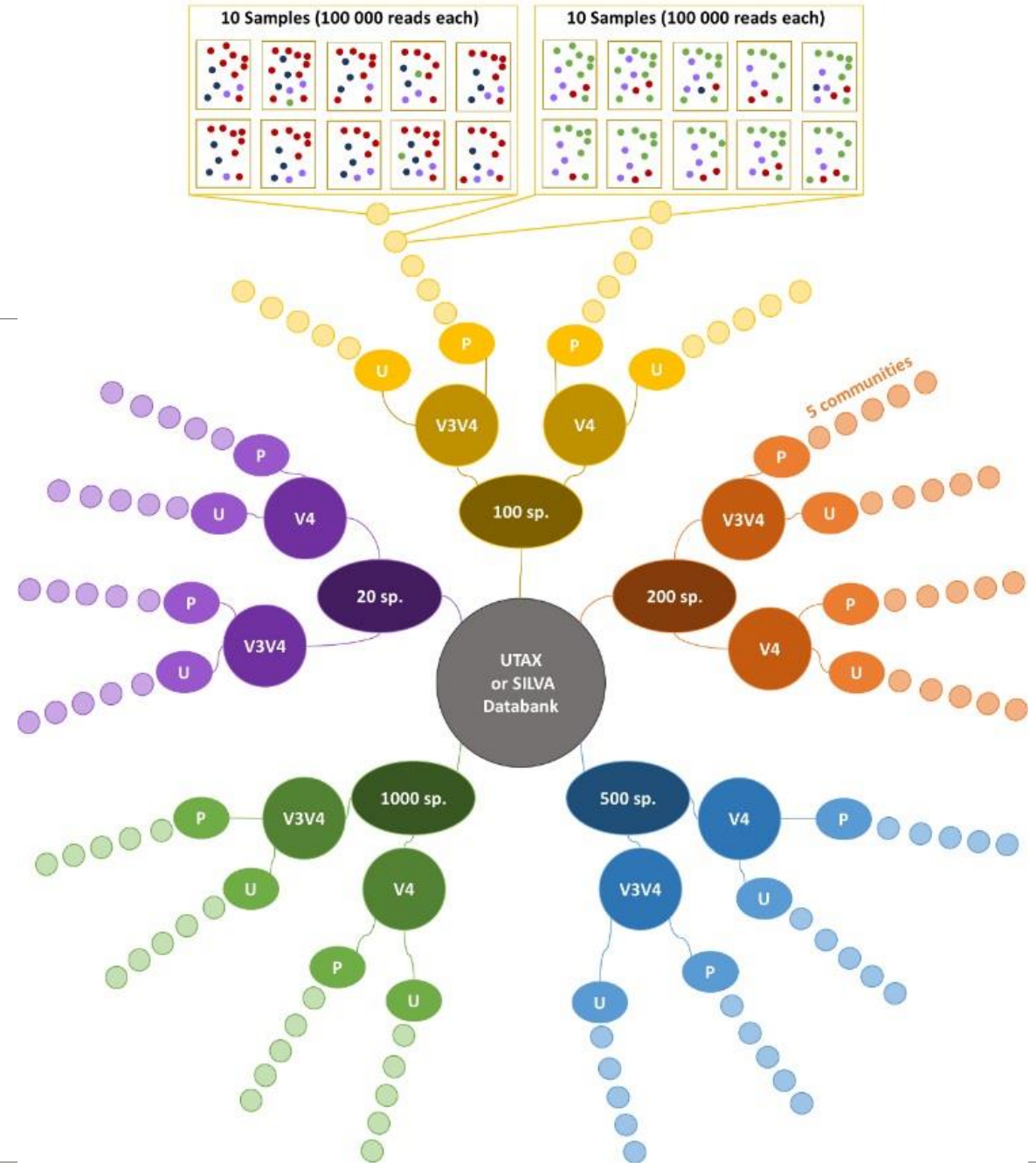
505 OTUs



FROGS' Accuracy






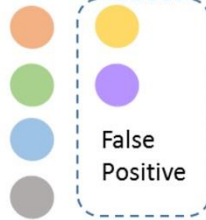
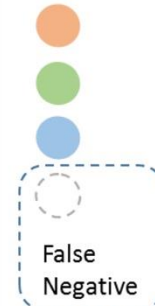
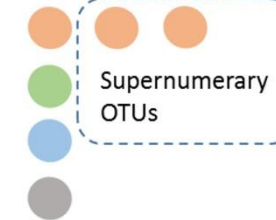
- 1.10^{+8} synthetic sequences were treated with **FROGS**, **UPARSE** and **MOTHUR**, **QIIME**, with their guidelines, to compare their performances
- 20, 100, 200, 500 or 1000 different species
- power law or a uniform distribution
- 5 to 20% of chimera

→ Divergence on the composition of microbial communities at the different taxonomic ranks



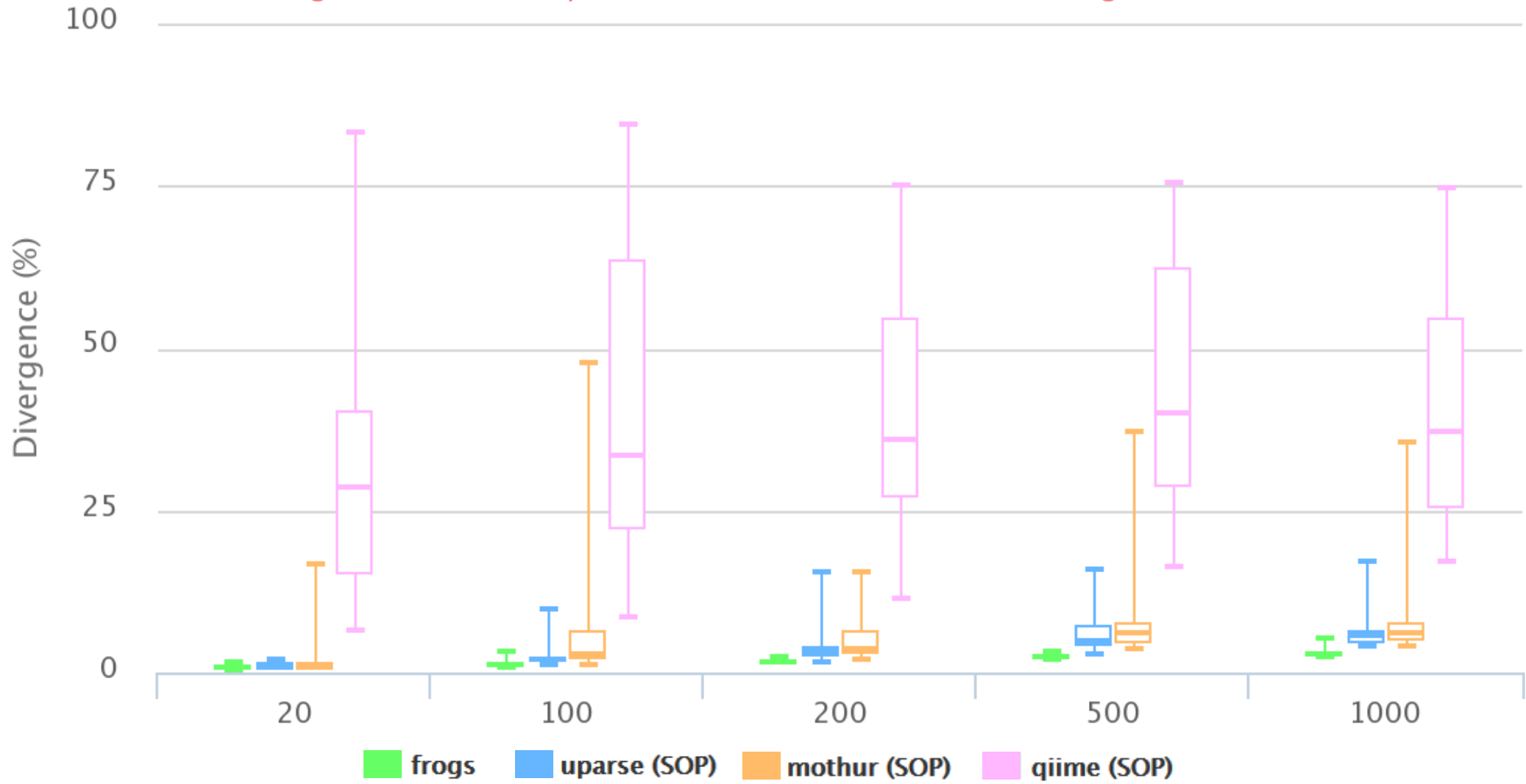
FROGS' Accuracy

The four metrics used to compare results of FROGS, UPARSE, QIIME and MOTHUR are :

<p>Expected</p>	 <p>1 species with abundance of 30 reads</p>	 <p>4 species</p>	 <p>4 species</p>	 <p>4 species</p>
<p>Observed</p>	 <p>divergence rate = +50% of abundance</p>	 <p>2 FPs = 2 not real OTUs are kept</p>	 <p>1 FN = a real OTU is lost</p>	 <p>2 SOs = 2 additional OTUs with same origin as the expected OTU</p>

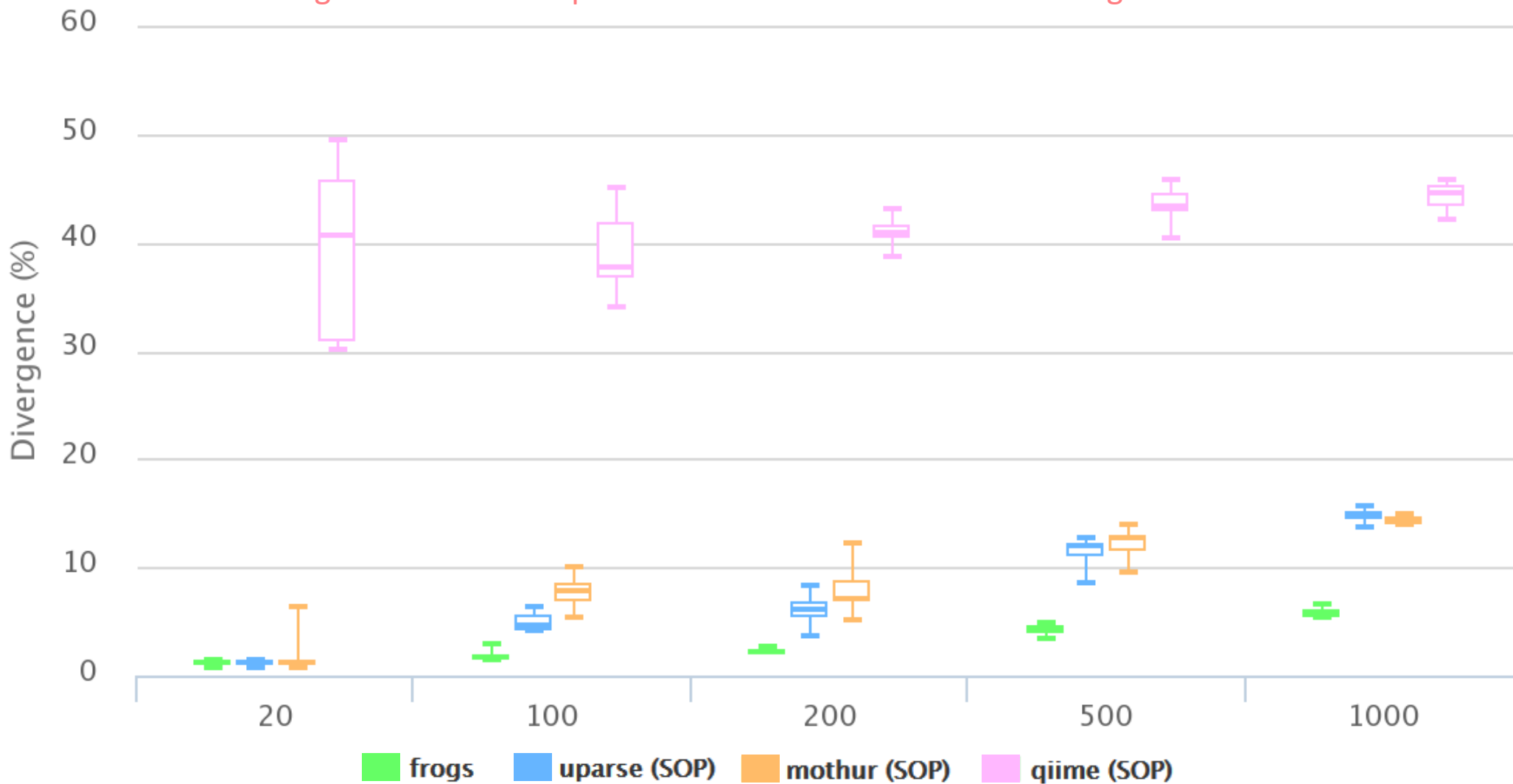
Affiliations divergence

Divergence on the composition of microbial communities at genus rank

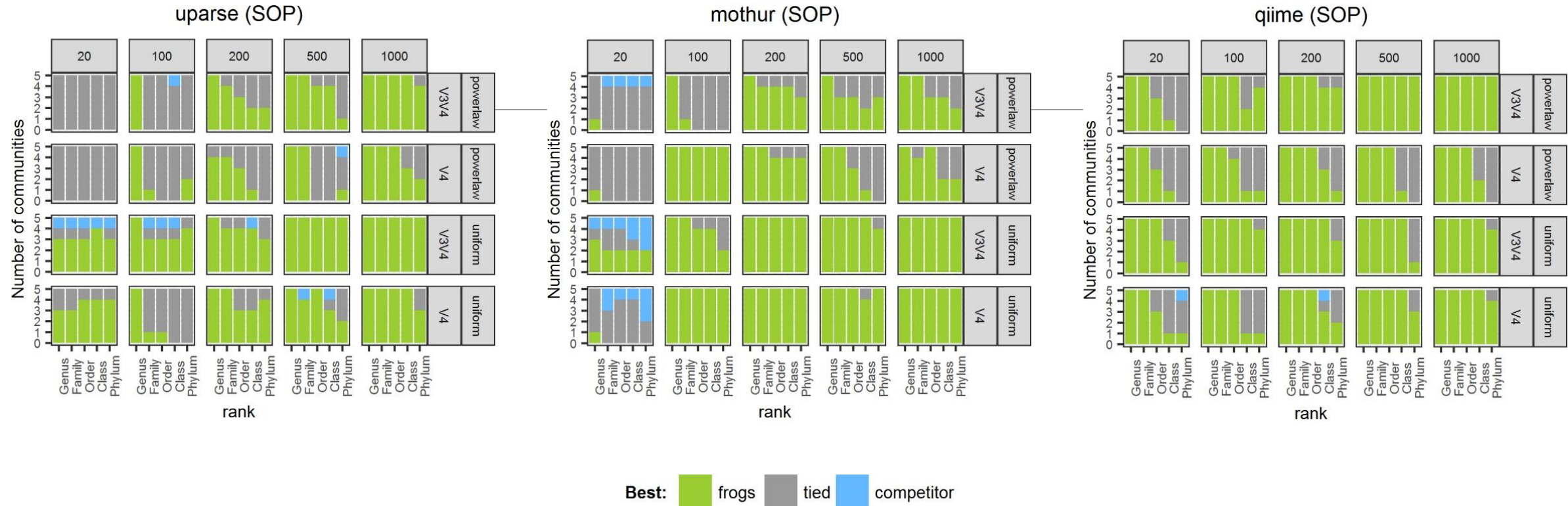


Affiliations divergence

Divergence on the composition of microbial communities at genus rank



The results of non-parametric paired tests (signed rank test) of Affiliation divergence on simulated data from UTAX

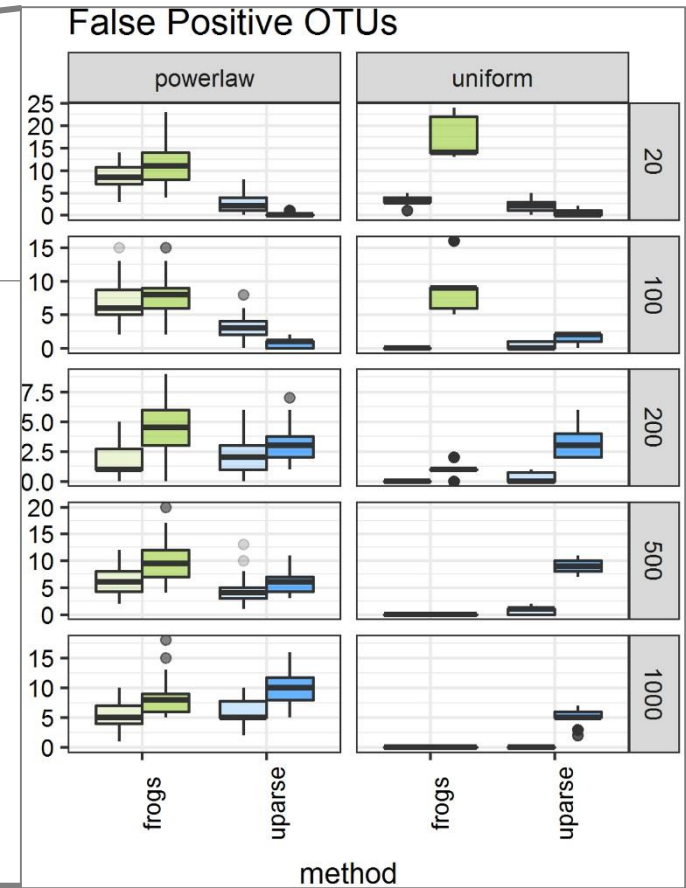
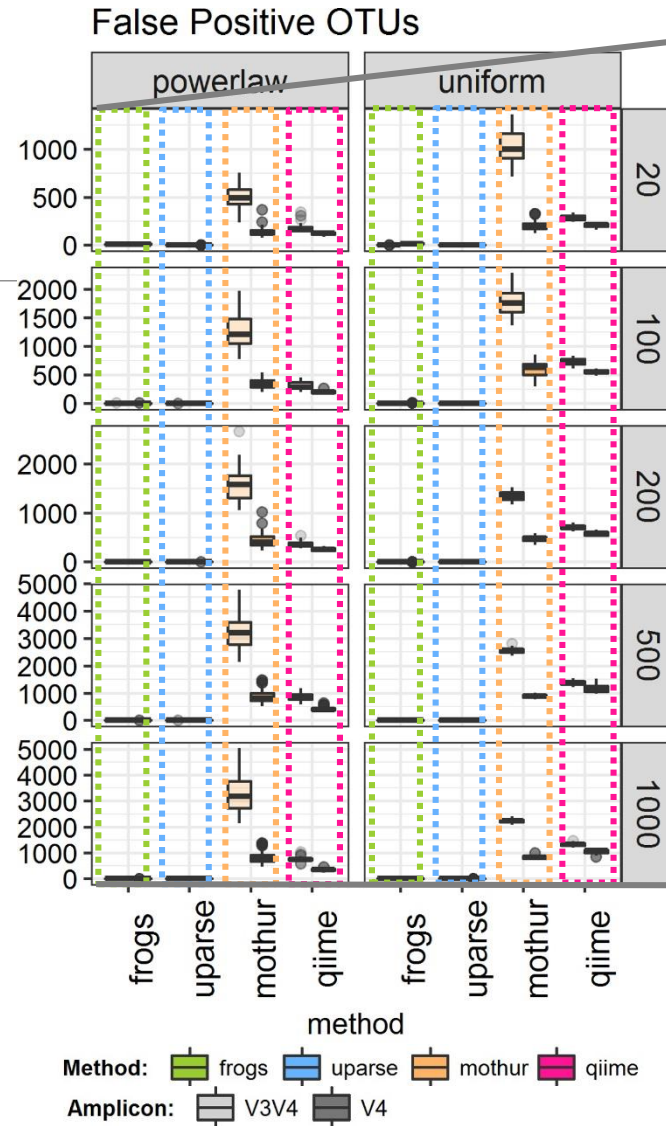


FROGS performed as well as or better than UPARSE and mothur in most settings. The infrequent condition in which FROGS performed worse than UPARSE and mothur was for small community sizes (20 species), except at genus level. It performed better than QIIME in all settings.

Huge number of FP inferred by mothur (up to 20 times more than the expected community size).

a few more FPs under power law abundance distributions and a few less under uniform abundance distributions (except for size < 100 species)

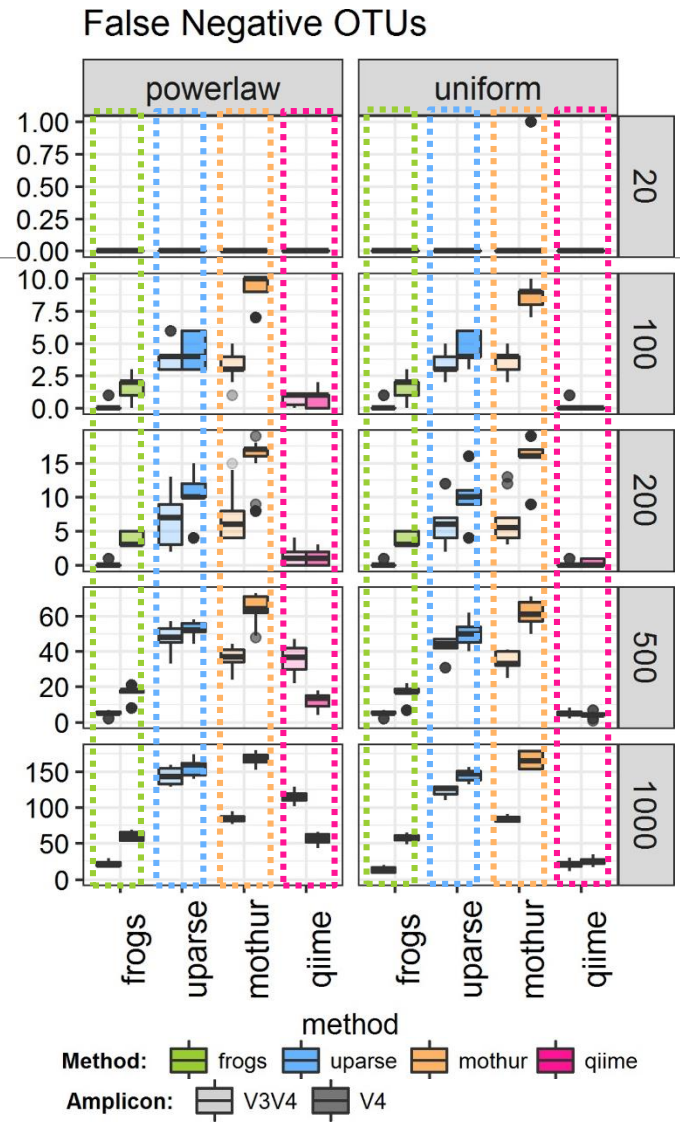
FROGS performed better than QIIME in all settings



FROGS truly outperformed mothur in terms of FN taxa

FROGS always produced fewer FNs than UPARSE.

FROGS sometimes produced more FNs than QIIME, especially on the V4 region.



Conclusions on assessments

FROGS performed much better than mothur in all settings

FROGS is less conservative than UPARSE for small size communities and better (for both FPs and FNs) for large size communities

FROGS is more conservative than QIIME on the V4 region and better (for both FPs and FNs) on V3V4 regions.

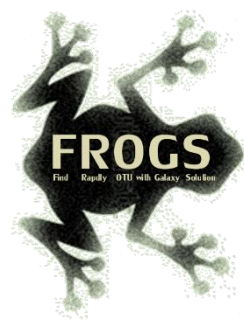
FROGS maintained both the number of FP and FN OTUs low, especially in complex communities.

→ cross-validation of chimeras, only used in FROGS, which avoids confusing real OTUs with chimeras.

→ 3 step strategy (clustering by Swarm + chimera removal with cross-validation + filtering) = a low FP rate and the high probability of detecting a species that is really present in the dataset *i.e.* a high recall rate.

→ unlike QIIME or mothur, FROGS never produced Supernumerary OTUs, which further validates the FROGS OTU picking strategy.

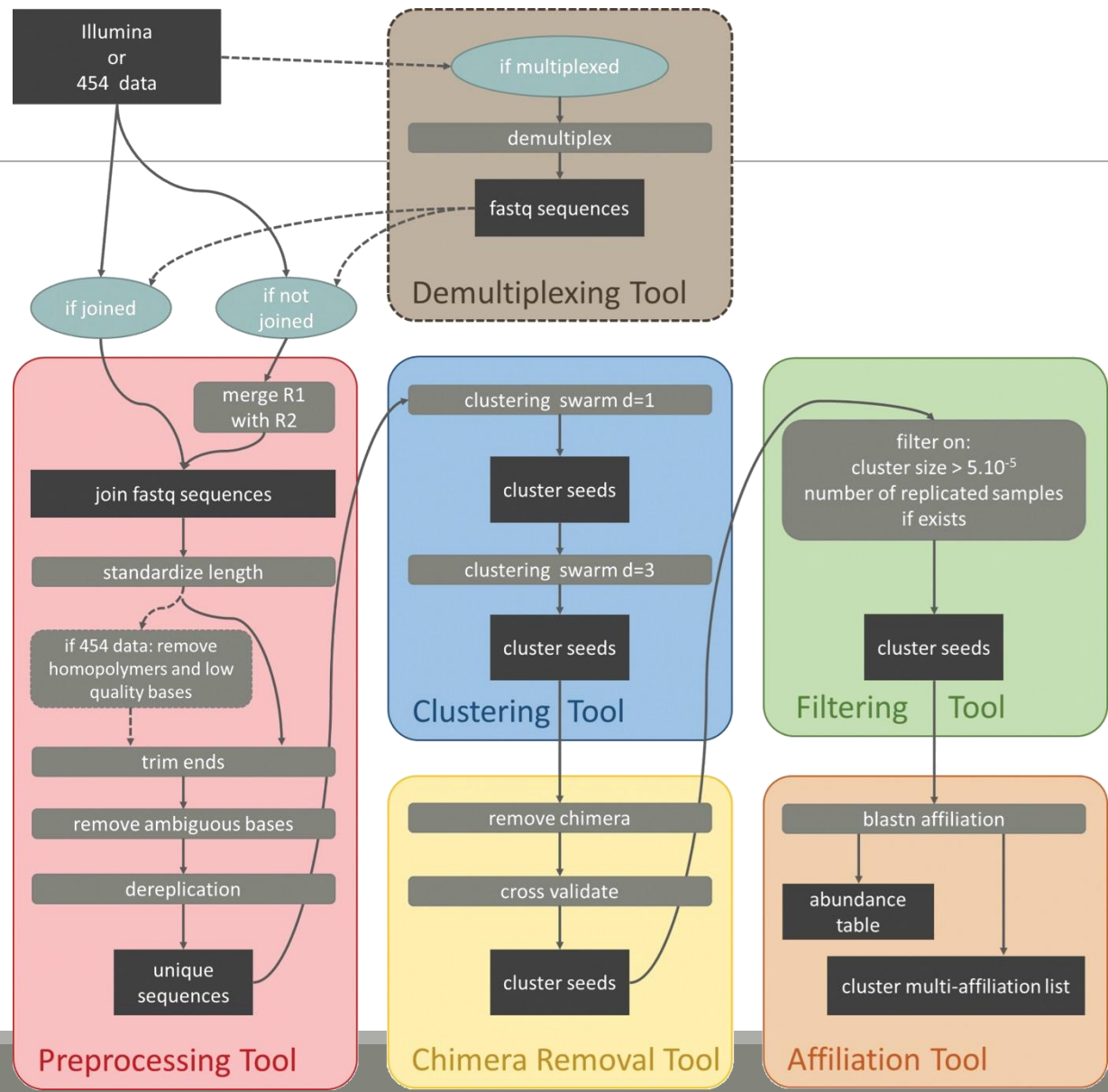
Conclusions

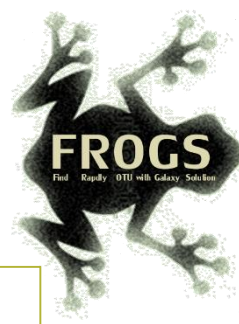


Why Use FROGS ?

- User-friendly
- Fast
- 454 data and Illumina data
- Clustering without global threshold and independent of sequence order
- Innovative chimera removal method (Vsearch + cross-validation)
- Filters tool
- Multi-affiliation with 2 taxonomy affiliation procedures
- Cluster Stat and Affiliation Stat tools
- Able to analyse ITS
- A lot of graphics
- Independant tools
- Few False Positives and few False Negatives

Our recommended guideline for mergeable reads:





How to cite FROGS

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Original Paper

OXFORD

Sequence analysis

FROGS: Find, Rapidly, OTUs with Galaxy Solution

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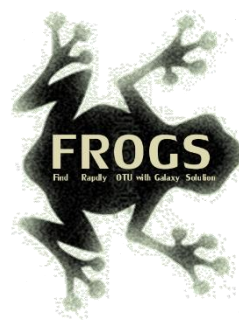
*To whom correspondence should be addressed.
[†]The authors wish it to be known that, in their opinion, the first two authors should be regarded as Joint First Authors.
Associate Editor: Bonnie Berger
Received on May 10, 2017; revised on December 1, 2017; editorial decision on December 4, 2017; accepted on December 5, 2017

Abstract
Motivation: Metagenomics leads to major advances in microbial ecology and biologists need user friendly tools to analyze their data on their own.
Results: This Galaxy-supported pipeline, called FROGS, is designed to analyze large sets of amplicon sequences and produce abundance tables of Operational Taxonomic Units (OTUs) and their taxonomic affiliation. The clustering uses Swarm. The chimera removal uses VSEARCH, combined with original cross-sample validation. The taxonomic affiliation returns an innovative multi-affiliation output to highlight databases conflicts and uncertainties. Statistical results and numerous graphical illustrations are produced along the way to monitor the pipeline. FROGS was tested for the detection and quantification of OTUs on real and *in silico* datasets and proved to be rapid, robust and highly sensitive. It compares favorably with the widespread mothur, UPARSE and QIIME.

Availability and implementation: Source code and instructions for installation: <https://github.com/geraldinepascal/FROGS.git>. A companion website: <http://frogs.toulouse.inra.fr>.
Contact: geraldine.pascal@inra.fr
Supplementary information: Supplementary data are available at *Bioinformatics* online.

1 Introduction
The expansion of high-throughput sequencing of rRNA amplicons has opened new horizons for the study of microbial communities. By making it possible to study all micro-organisms from a given environment without the need to cultivate them, metagenomics has led to major advances in many fields of microbial ecology, from the study of the impact of microbiota on human and animal pathologies (Hess *et al.*, 2011; Hooper *et al.*, 2012; Jovel *et al.*, 2016) to the study of biodiversity in environmental ecosystems and the search for biomarkers of pollution (Andres and Bertin, 2016; de Vargas *et al.*, 2015). Determining the composition of a microbial ecosystem, at low cost and great depth, is still largely based on the amplification and sequencing of biodiversity marker genes, also called amplicons, such as rRNA genes and ITS. The clustering of sequences into

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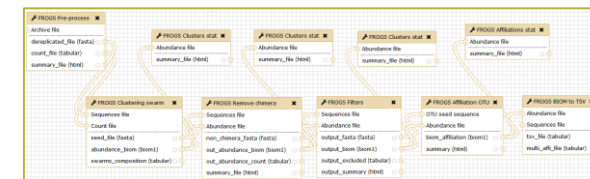
FROGS'docs

Website: <http://frogs.toulouse.inra.fr>

Tuto: <https://youtu.be/Kh6ZrlmKGoY>

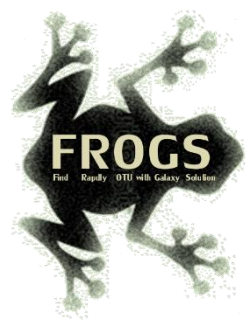
Pipeline FROGS on

<http://sigenae-workbench.toulouse.inra.fr/galaxy/u/gpascal/w/to-test-frogs>



All scripts on Github: <https://github.com/geraldinepascal/FROGS.git>





To contact

FROGS:

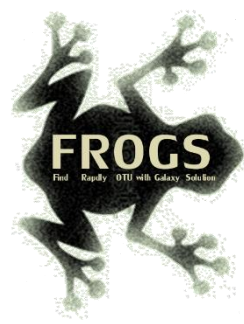
frogs@inra.fr

Galaxy:

support.sigenae@inra.fr

Newsletter – subscription request:

frogs@inra.fr



Play list FROGS:

https://www.deezer.com/fr/playlist/5233843102?utm_source=deezer&utm_content=playlist-5233843102&utm_term=18632989_1545296531&utm_medium=web